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PTEN and SUMOylation in Membrane Protein Trafficking

PTEN and SUMOylation in Membrane Protein Trafficking

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Abstract

The lipid phosphatase PTEN is most well-known for its role in dephosphorylating PIP_3 to negatively regulate the Akt pathway. This pathway is important in tumour suppression, and has more recently been shown to be involved in synaptic plasticity. PTEN is mutated in various cancers and dysregulated in Alzheimer's Disease, suggesting it may be a useful therapeutic target.

PTEN activity is controlled by a range of post-translational modifications (PTMs) including SUMOylation, which has been reported to regulate the localisation, function, stability and coupling to the Akt pathway.

The initial aim of this study was to assess if, and how, PTEN SUMOylation affects its reported functions. To achieve this, I optimised protocols to detect SUMOylation and characterise a novel "PTEN-3KR" mutant, in which all three known SUMO acceptor lysine residues are substituted to arginine.

Unexpectedly, PTEN-3KR displayed both enhanced SUMOylation and ubiquitination. While surprising, this provided an interesting tool to investigate the consequences of these increased levels of PTEN modification. I found that PTEN-3KR is unable to supress the Akt pathway, but its turnover and ability to dimerize were unaffected. I also investigated how PTEN-3KR impacted on AMPAR trafficking and retromer function. PTEN-WT, but not PTEN-3KR, overexpression upregulated total protein levels of retromer components SNX27 and VPS26. Compared to PTEN-WT, PTEN-3KR also bound less to SNX27 and decreased surface expression of a prototypic retromer cargo GluT1.

These results confirm the importance of SUMOylation and/or ubiquitination in the regulation of PTEN activity. Moreover, they demonstrate a novel role for SUMOylation of PTEN in regulating the retromer complex and protein trafficking.

Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Acknowledgements

This thesis is dedicated to my father Neil Milligan, who taught me if you believe in yourself and work hard you can make your dreams come true.

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Abbreviations

AD Alzheimer's Disease

AMPAR α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor

ATP Adenosine Triphosphate

BSA Bovine Serum Albumin

CBP Calmodulin Binding Peptide

DAPI 4′,6-diamidino-2-phenylindole

DIV Days in vitro

FBS Foetal Bovine serum

GFP Green Fluorescent Protein

HEK Human Embryonic Kidney

MEF Mouse Embryonic Fibroblast

NMDAR N-Methyl-D-Aspartate Receptor

NEM N-Ethylmaleimide

PD Parkinson's Disease

PLL Poly L-Lysine

PTEN Phosphatase and Tensin Homolog

PTM Post Translational Modification

PTSD Post Traumatic Stress Disorder

PSD-95 Post Synaptic Density Protein

Ran-Gap RAS-related Nuclear GTPase activating Protein

RNF4 RING Finger Protein 4

RT Room Temperature

SAE SUMO Activating Enzyme

SBP Streptavidin Binding Peptide

SDS Sodium Dodecyl Sulphate

SDS-PAGE Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SENP Sentrin/SUMO-specific proteases

shRNA Short Hairpin RNA

SIM SUMO Interacting Motif

STUbL SUMO targeted Ubiquitin Ligase

SUMO Small Ubiquitin like Modifier

UBC9 Ubiquitin Conjugating Enzyme E2I

WT Wildtype

List of Figures

List of Tables

General Introduction

1 General Introduction

1.1 Synaptic Plasticity

1.1.1 Overview- PTEN, Plasticity, Post Translational Modifications and Retromer

Alterations to synaptic transmission in response to activity or experience are referred to as synaptic plasticity (Citri and Malenka 2008; Larrabee 1947; Hughes 1958). This process is reliant on changes to the biophysical properties of the synapse, including alternations in the trafficking of neuronal receptors; this fundamental process underlies mechanisms of learning and memory (Collingridge *et al*. 2004; Beattie *et al*. 2000). Post translational modifications (PTMs) such as SUMOylation of neuronal proteins have been shown to influence plasticity (Jaafari *et al*. 2013). Phosphatase and Tensin Homolog (PTEN) is critical in plasticity (Jurado 2017), and is reported to play a role in Alzheimer's disease-related pathology (Knafo *et al*. 2016). PTEN can also be SUMOylated and ubiquitinated (Trotman *et al*. 2007; Huang *et al*. 2012), however, the role of PTEN SUMOylation in neurons and plasticity has not been assessed. Therefore, this thesis aims to further examine the role of PTEN in plasticity with a focus on receptor trafficking, and establish whether SUMOylation plays a role. I will also assess the influence of PTEN on the retromer complex, a trafficking complex which regulates endosome to cell surface trafficking of membrane proteins including neurotransmitter receptors (Vagnozzi and Pratico 2019; Temkin *et al*. 2017; Seaman 2007; Gallon and Cullen 2015). It has been reported that PTEN can interrupt retromer formation and influence membrane protein trafficking in cell lines, but this is not well characterised (Shinde and Maddika 2017). The introduction of this thesis will outline the fundamental cell biology behind plasticity, PTEN, SUMOylation and retromer, then explain their involvement to relevant diseases.

1.1.2 The Synapse

Neurotransmitters are passed between a neuron and its neighbouring cell at a specialised junction called a synapse (Harris and Littleton, 2015). Chemical synaptic transmission involves the delivery of neurotransmitters from the pre-synapse, facilitated by calcium influx after an action potential (Katz and Miledi 1968, 1967b; Sudhof *et al*. 2012; Sheffler 2020). This causes neurotransmitter-containing vesicles to fuse with the membrane of the pre-synapse and release the neurotransmitters into the synaptic cleft and detection by receptors at the postsynaptic membrane (Sheffler 2020; Sudhof *et al*. 2012) (Figure 1.1.2.1). There are many kinds of neurotransmitter including dopamine, serotonin, acetylcholine and glutamate (Sheffler, 2020). This work will primarily focus on glutamatergic transmission, which is the

neurotransmitter found most abundantly in the brain, and underpins various mechanisms of learning and memory (Zhou and Danbolt 2014; Barco *et al*. 2006). Approximately 80% of glutamate found in neurons in synthesized by an enzyme called Phosphate-Activated Glutaminase (PAG), which converts glutamine into glutamate by removing its amido group (Nadler, 2012; Schousboe et al. 2014). In astroglia, glutamate is converted back into glutamine by condensation of glutamate and ammonia by Glutamine Synthetase (GS) (Martinez-Hernandez, 1997; Shen, 2013).

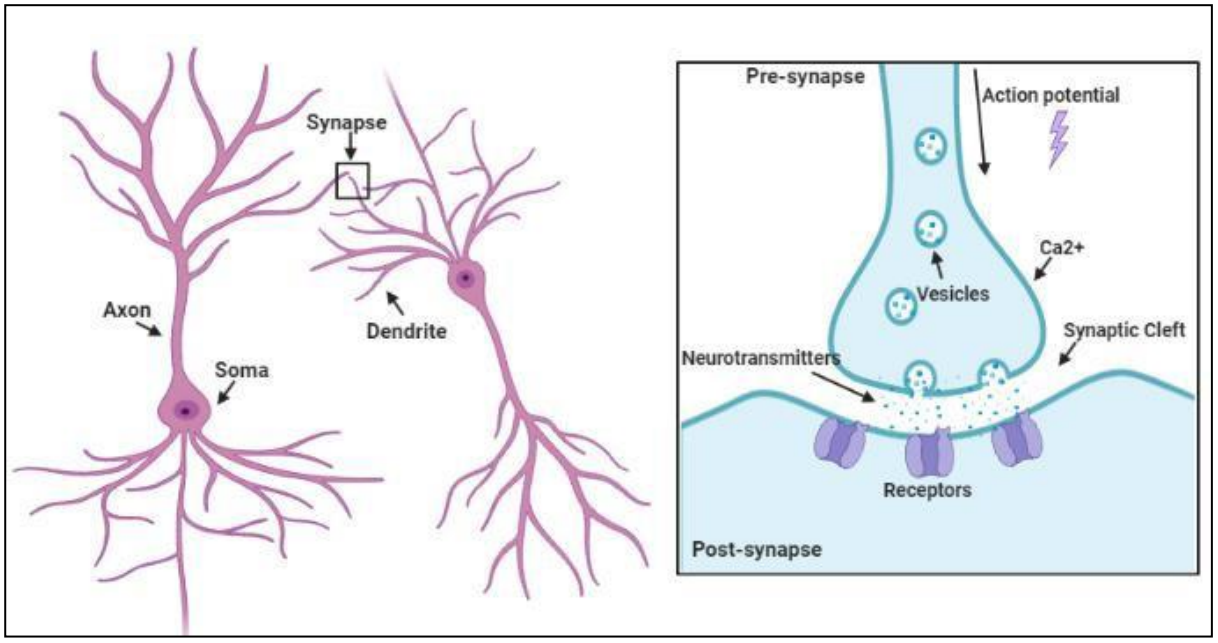

Figure 1.1.2.1. The Synapse.

This schematic depicts hippocampal neurons and a chemical synapse, adapted from (Lodish 2000; Mabb and Ehlers 2010). The diagram on the left shows two neurons, with the axon, soma (cell body), synapse and dendrite labelled. Dendrites form synapses with other neurons, enabling them to receive signals released by axons which conduct the electrical impulse (Lodish 2000). On the right, the diagram shows a chemical synapse. When an action potential causes depolarisation, calcium floods the cell, causing vesicles to fuse with the presynaptic membrane and exocytose neurotransmitters into the synaptic cleft, which are picked up by receptors at the post-synapse (Katz and Miledi 1967a; Sudhof 2012). Schematic created in Biorender.com, using premade neuron and synapse shapes.

1.1.3 Glutamate Signalling

Glutamate receptors are able to mediate glutamate signals in processes involved in learning and memory (Barco, Bailey, and Kandel 2006). Glutamate signalling provides the majority of fast, excitatory transmission in the brain, and is mediated by four kinds of glutamate receptors: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs), *N*-methyl-D-aspartate receptors (NMDARs), Kainate receptors and metabotropic receptors

(Hollmann and Heinemann 1994; Zhou and Danbolt 2014; Fleming and England 2010). AMPARS, NMDARs and Kainate receptors are known as ionotropic receptors, which are ligated-coupled ion channels that pick-up glutamate signals released from the pre-synapse (Martinez-Lozada *et al*. 2015; Greger *et al*. 2017). Changes to AMPAR receptor levels at the cell surface are fundamental in plasticity (Collingridge *et al*. 2004; Beattie *et al*. 2000).

1.1.4 AMPAR Subunits

AMPARs function as heteromers, with different combinations of four primary subunits GluA1-4 (Wenthold *et al*. 1992; Dingledine *et al*. 1999; Hollmann and Heinemann 1994) (See Figure 1.1.5.1. for schematic of AMPAR topology). Each subunit has a different role in ion selectivity and trafficking properties, so the ability of AMPARs to heterodimerize leads them to have functional variation (Lu *et al*. 2009; Greger *et al*. 2017). For example, different combinations of these subunits give them different cation channel properties; the presence of the GluA2 subunit causes Na^+ permeability, AMPARs are not Na^+ permeable unless they have this subunit (Sommer *et al*. 1991; Martinez-Lozada and Ortega 2015). Lu *et al*. (2009) found the dominant heteromers are GluA1 and GluA2 at CA1 synapses in the hippocampus, making up about 80% of the population of AMPARs. In pyramidal neurons in CA1, all surface AMPARs contain GluA2. Lu *et al*. (2009) also found cells in which GluA1, A2 and A3 are knocked out do not have AMPAR EPSCs at synapses, but NMDARs EPSCs are unchanged. In CA1, the majority of synaptic transmission is regulated by GluA1/2 heteromers; GluA2 KO in mice leads to a 50% loss of AMPAR mEPSCs and causes a dramatic change in frequency, without an effect on amplitude. Therefore, Lu *et al*. (2009) suggest around half of the synapses have no AMPAR mediated activity under GluA2 KO, and some AMPARs containing GluA2 are gradually replaced by AMPARs without GluA2. This suggests there are two populations of synapses which vary depending on whether they are able to engage GluA2 lacking receptors (Lu *et al*. 2009). Deleting GluA3 simultaneously with GluA2 had little extra effect, suggesting GluA3 is more dispensable for transmission (Lu *et al*. 2009).

1.1.5 AMPAR Q/R Editing Site

The majority of AMPARs in the adult brain are calcium-impermeable, due to replacement of Q607 with an arginine through RNA editing in the pore loop of the GluA2 subunit (Henley and Wilkinson 2016; Greger *et al*. 2003; Sommer *et al*. 1991) (see Figure 1.1.5.1. for schematic of channel conductance after AMPAR Q/R editing). This RNA editing also regulates exit from the endoplasmic reticulum (ER) and tetramerization; unedited subunits tetramerise and are trafficked to the cell surface, while subunits which have been edited to arginine localise mostly in the ER and are largely dimeric (Greger *et al*. 2003).

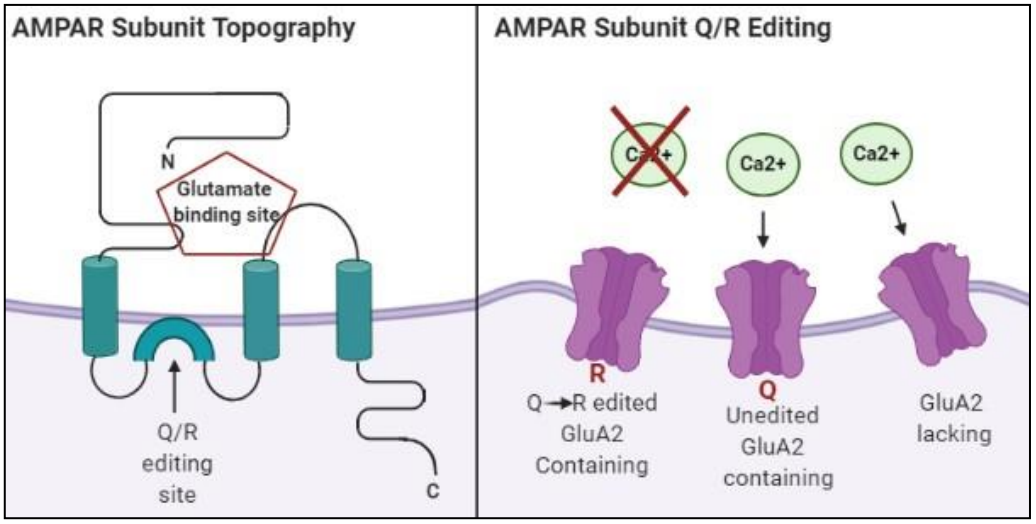

Figure 1.1.5.1. AMPA Receptor Topology and Q/R Editing.

This schematic on the left depicts an AMPAR subunit, showing its intracellular re-entrant loop with Q/R editing site (in GluA2 subunit), transmembrane domains and glutamate binding site. Schematic on the right shows three states of AMPARs in regards to calcium conductance. Unedited and GluA2 lacking AMPARs allow calcium to flow into the cell, those with the Q607 site in the pore loop edited to arginine through RNA editing do not allow calcium entry (Henley and Wilkinson 2016; Greger *et al*. 2003; Sommer *et al*. 1991). Schematic adapted from Henley and Wilkinson (2016), Widagdo *et al*. (2017) and Wright and Vissel (2012) and created in biorender.com with premade shapes.

1.1.6 Short Term Synaptic Plasticity

The mechanism by which synapses change their properties in response to the level of stimulation they experience is termed synaptic plasticity (Larrabee 1947; Citri and Malenka 2008; Hughes 1958). Broadly speaking, synaptic plasticity can refer to synaptic pruning, neurogenesis, or activity-Dependent changes in synaptic strength (Barco, Bailey, and Kandel 2006); this thesis will focus on the latter.

The history of activation of the synapse can affect the probability/magnitude of the neurotransmission at the next stimulation, which is referred to as paired-pulse facilitation (PPF) or depression depending on the direction of the probability of release (Jackman and Regehr 2017; Citri and Malenka 2008; Katz and Miledi 1968). After stimulation of excitatory synapses, the chance of a second neurotransmission event is initially enhanced, up to a point at which the probability of release drops off (Citri and Malenka 2008; Katz and Miledi 1967b, 1968). This is because the activity temporarily increases calcium in the presynapse, which can facilitate the next potential (Citri and Malenka 2008; Katz and Miledi 1968; Dobrunz and Stevens 1997). Specifically, the probability of PPF at the synapse is inversely related to the probability of the starting release probability and directly correlates with the amount of neurotransmitter available for release (Dobrunz and Stevens 1997).

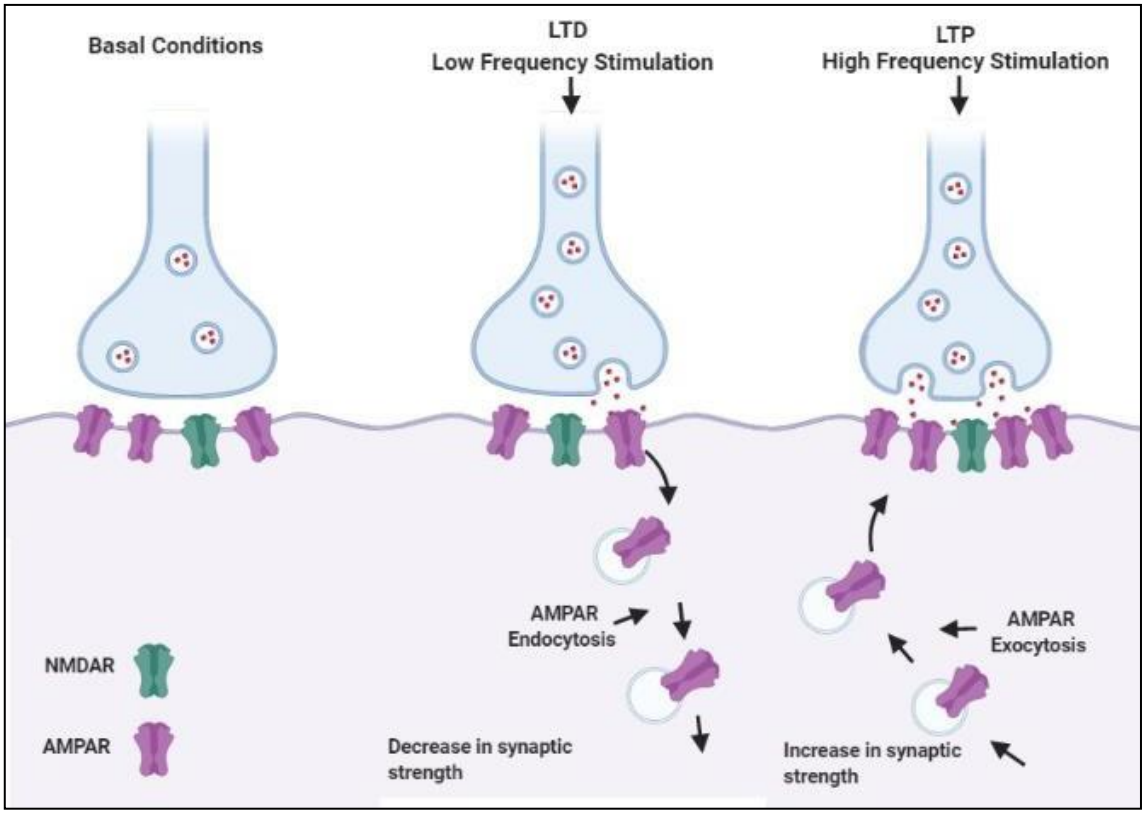

Figure 1.1.6.1 AMPA Receptor Trafficking in Long Term Depression and Long Term Potentiation.

This schematic depicts an unstimulated synapse on the left; AMPARs will be constitutively trafficking to and from the membrane in these conditions (Henley and Wilkinson 2013; Chen and Maghsoodi 2007). The central panel shows increased AMPAR endocytosis in response to low frequency stimulation, leading to a decrease in synaptic strength; the right-hand panel shows increased AMPAR trafficking to the synapse after high frequency stimulation, increasing synaptic strength (Citri and Malenka 2008; Beattie *et al*. 2000; Collingridge, Isaac, and Wang 2004; Fleming and England 2010; Carroll *et al*. 1999; Chen and Maghsoodi 2007). This schematic is adapted from Chen and Maghsoodi (2007), Citri and Malenka (2008), and OpenstaxCNX (2016) and created in biorender.com using premade shapes.

1.1.7 Long Term Synaptic Plasticity

There are two opposite processes which coordinate long-term synaptic response to different types of stimulation. The first is termed Long Term Potentiation (LTP), where synapse response probability is increased following high-frequency stimulation (Bliss and Lomo 1973; Ho 2011). The second is called Long Term Depression (LTD), which occurs following low frequency stimulation and is characterised by weakening of synaptic responses (Lynch, Dunwiddie, and Gribkoff 1977; Ho 2011; Dudek and Bear 1992). These processes can be measured by electrophysiological recordings in mouse brain hippocampal slices; synaptic activity as measured by excitatory post-synaptic potentials (EPSPs) shows a sustained

increase or decrease after high or low stimulation (Ho 2011). LTP was first discovered in the rabbit hippocampus, where following stimulation, synaptic responses were potentiated for up to 10 hours (Bliss and Lomo 1973).

There are two main mechanisms by which these changes in synaptic strength are created; changes to level of neurotransmitter release from the pre-synapse to the post synapse, and changes in the amount and activity of receptors which detect the neurotransmitters (Fleming and England 2010). Regarding changes to receptors, this is partly due to changes in AMPAR levels at synapses; LTD leads to removal of AMPARs and subsequent synaptic depression, while LTP leads to insertion of more AMPARs and therefore increased excitability (Beattie *et al*. 2000; Collingridge *et al*. 2004; Fleming and England 2010; Carroll *et al*.1999) (See Figure 1.1.2.1.).

1.1.8 NMDAR-Dependent Plasticity

Synaptic plasticity is often studied in the hippocampus, an area of the brain where information flows in a loop (Neves *et al*. 2008). NMDAR-dependent LTD and LTP are most well characterised in the hippocampus; this area has a high level of NMDAR expression and is critical for spatial learning and memory (Nakazawa *et al*. 2004; Malenka and Bear, 2004). AMPARs and NMDARs are colocalised at excitatory hippocampal synapses; approximately 70% of synapses contain both kinds of receptor (Bekkers and Stevens, 1989).

NMDARs and AMPARs are ligand-gated ion channels; NMDARs require both transmitter release and depolarisation for activation, they are calcium permeable and mediate calcium influx needed for LTP induction when activated (Ho, 2011). When AMPARs are activated by glutamate binding, there is partial depolarisation of the neuron due to sodium influx through AMPARs, leading to the removal of the voltage-Dependent magnesium block from co-localised NMDARs (Bekkers and Stevens 1989; Greger *et al*. 2017; Watson 2012; Luscher and Malenka 2012; Mayer 1984; Nowak *et al*. 1984; Nakazawa *et al*. 2004; Molnar 2019). This allows calcium influx through voltage gated calcium channels and NMDARs and increased depolarisation, leading to changes in the level of AMPARs at the membrane and modulation of synaptic strength (Bliss and Collingridge 1993; Watson 2012; Mayer 1984; Nowak *et al*. 1984; Greger *et al*. 2003; Greger *et al*. 2017; Hunt and Castillo 2012; Luscher and Malenka 2012).

Synaptic NMDAR activation increases both AMPAR and NMDAR surface expression, but extra-synaptic NMDAR activation decreases surface expression of both proteins (Li *et al*.

2009). Through this mechanism, NMDARs can regulate plasticity through regulation of AMPAR postsynaptic expression, this is one of the main processes underpinning learning and memory (Collingridge *et al*. 1983; Morris *et al*. 1986; Dudek and Bear 1992; Li *et al*. 2009; Bashir *et al*. 1991). NMDARs are critical in LTP; since the NMDAR antagonist AP5 blocks LTP without effecting basal synaptic transmission (Collingridge, *et al*. 1983). Specifically, AP5 application leads to selective inhibition of hippocampal-dependent place learning, without effecting retention of spatial information already learnt, suggesting NMDARs are critical in spatial learning (Morris *et al*. 1986).

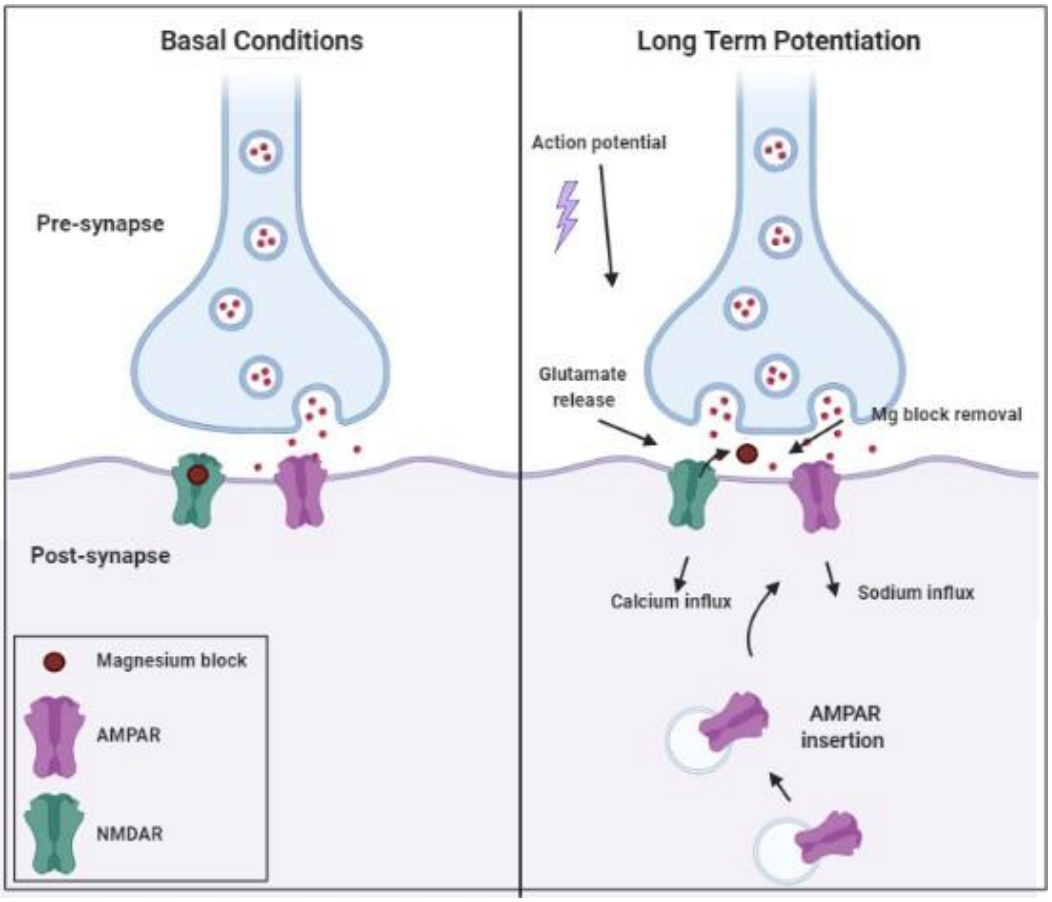

Figure 1.1.8.1 NMDAR-Dependent LTP.

When AMPARs are activated by glutamate binding, sodium ions flow into the post synapse, causing partial depolarisation and removal of the magnesium block from NMDARs (Watson 2012; Mayer 1984; Nowak *et al*. 1984; Luscher and Malenka 2012). This causes calcium influx and increased depolarisation, leading to changes in the level of AMPARs at the membrane and modulation of synaptic strength (Bliss and Collingridge 1993; Watson 2012; Nakazawa *et al*. 2004; Molnar 2019; Luscher and Malenka 2012). Schematic adapted from Molnar and Gair (2019) and Watson and Breedlove (2012). Created in biorender.com using premade shapes.

1.1.9 AMPAR Trafficking

AMPARs are subject to both constitutive and activity-dependent movement to and from synapses, referred to as AMPAR trafficking (Henley and Wilkinson 2013). Trafficking of AMPARs is important for maintaining synaptic efficacy (Shi *et al*. 2001). AMPAR trafficking is regulated by transport via molecular motor proteins such as dynein, which is a cytoskeletal motor protein that moves cellular cargoes around the cell (He *et al*. 2005; Levy and Holzbaur 2006; Kapitein *et al*. 2010). For example, dynein motors drive axonal transport and move AMPARs into dendrites (Kapitein *et al*. 2010). Inactivation of dynein reduces AMPAR EPSCs, again suggesting the role of dynamin in AMPAR transport (Kim and Lisman 2001).

Variation in which subunits AMPARs contain gives rise to differences in the way they are trafficked (Shi *et al*. 2001). During stimulation, GluA1 and 2 are inserted into synapses, while heteromers containing GluA2 and 3 are constitutively trafficked to synapses (Shi *et al*. 2001; Passafaro *et al*. 2001). Local trafficking to and away from the surface occurs via recycling endosomes and sustains AMPARs at synapses which is critical in plasticity; blocking this movement though endosomes inhibits LTP (Park *et al*. 2004).

AMPAR trafficking is also influenced by various post-translational modifications including phosphorylation, palmitoylation and ubiquitination (Boehm *et al*. 2006; Henley and Wilkinson 2013; Lin *et al*. 2009; Widagdo *et al*. 2015). Phosphorylation plays a key role in regulating AMPAR insertion and removal; for example, phosphorylation of AMPAR subunit GluA1 at serine 818 by Protein Kinase C (PKC) controls AMPAR insertion during LTP (Boehm *et al*. 2006; Henley and Wilkinson 2013). Blocking phosphorylation at this site limits LTP and inhibits PKC-induced membrane insertion of GluA1 (Boehm *et al*. 2006) GluA1 dephosphorylation is generally associated with endocytosis and LTD (Henley and Wilkinson 2013).

1.1.10 AMPARs, TARPs and Scaffolding Proteins

Scaffolding proteins play a crucial role in synaptic transmission through tethering receptors, stabilising them and building signalling complexes (Elias and Nicoll 2007; Garbett and Bretscher 2014). Transmembrane AMPA Receptor Regulatory Proteins (TARPs) are proteins which associate with AMPARs and regulate their expression, anchoring at the synapse, conformation and activity (Carbone and Plested 2016; Chen *et al*. 2000; Priel *et al*. 2005; Schnell *et al*. 2002; Bats *et al*. 2007).

Stargazin, a suggested auxiliary subunit of AMPARs (Vandenberghe *et al*. 2005), can associate with all four AMPAR subunits and facilitates their delivery to the cell

surface (Chen *et al*. 2000). Stargazer mice, which do not express Stargazin, express AMPARs but these localise at synapses dramatically less than in Stargazin heterozygous mice (Chen *et al*. 2000; Hashimoto *et al*. 1999). Stargazer mice also cannot produce AMPAR EPSCs under basal conditions and show ataxia (Chen *et al*. 2000; Hashimoto *et al*. 1999). The ability for Stargazin to interact with AMPARs is critical for surface delivery of AMPARs, while Stargazin interactions with postsynaptic density protein of 95 kDa (PSD-95) is critical for AMPAR targeting to synapses (Chen *et al*. 2000). Rescue of Stargazin expression in Stargazin knockout granule cells rescues AMPAR currents; this is mediated by Stargazin binding to PSD-95, as expression of Stargazin mutant which lacks its amino-terminal PSD-95, Disc-large and ZO-1 (PDZ) binding domain does not rescue AMPAR currents (Chen *et al*. 2000). Expression of this mutant, which cannot localise at synapses in hippocampal neurons, also reduces hippocampal AMPAR miniature EPSCs compared to untransfected neurons (Chen *et al*. 2000). Other work has confirmed that interactions between PSD-95 and Stargazin regulate movement of AMPARs between synaptic and extra synaptic sites, and disruption this interaction destabilises AMPARs, reducing AMPAR clustering at the post-synapse (Bats *et al*. 2007). In addition to surface expression, Stargazin can also module AMPAR conductance (Carbone and Plested 2016). Stargazin can exist in two states: an inactive state during basal conditions, and an active state which increases the channel conductance of AMPARs, driven by AMPAR channel opening (Carbone and Plested 2016).

Expression of a mutant of an endosomal scaffolding protein, Eps15–homology domain protein EHD1/Rme1 (Rme1-G429R) (known to act as dominant negative) causes slowing of receptor recycling from endosomes towards the plasma membrane (Park *et al*. 2004; Lin *et al*. 2001; Grant and Caplan 2008). When trafficking from endosomes to the plasma membrane was prevented by expression of this mutant, AMPARs accumulated intracellularly. There was a re-localisation of the total population of AMPARs from spines to endosomal compartments in dendritic shafts (Park *et al*. 2004). This mutant was also able to block LTP induction, evidenced by a lack of AMPAR insertion after glycine stimulation (Park *et al*. 2004). Park *et al*. (2004) discovered that AMPARs inserted during LTP are not newly synthesised, but come from a recycled pool, highlighting the importance of the endosomal recycling pathway and receptor trafficking in synaptic functioning and plasticity.

1.1.11 Glutamate Receptor-Interacting Proteins (GRIPs)

GRIPs can interact with AMPARs through their PDZ domain and are involved in AMPAR trafficking and clustering at synapses (Bissen *et al*. 2019; Dong *et al*. 1997). GRIP1 is an adaptor protein which links AMPARs to other proteins involved in transport such as E-cadherin and motor protein kinesin superfamily (KIF) protein, promoting AMPAR transport to dendrites (Heisler *et al*. 2014; Dong *et al*. 1997). GRIP1 is described by Heisler *et al.* (2014) as a "*multilink interface*", which is used by KIF5 to promote N- cadherin/GluA2 transport to dendrites, a process involved in stabilisation of excitatory synapses and dendritic spine structure. GRIP1 is reported to regulate both insertion and removal of AMPARs into the synaptic membrane (Bissen *et al*. 2019; Osten *et al*. 2000; Hirbec *et al*. 2003). GRIP1 binding to AMPARs is also negatively regulated by phosphorylation by PKC (Matsuda *et al*. 1999). These studies show that various neuronal proteins such as PSD-95, GRIP1 and Stargazin and their interactions with AMPARs are necessary for correct synaptic function and reflect the complexity of neuronal processes (Bissen *et al*. 2019; Dong *et al*. 1997; Chen *et al*. 2000).

1.2 PTEN

1.2.1 Overview-PTEN

Phosphatase and Tensin Homolog (PTEN), is a phosphatase with tumour suppressive activity, encoded by the *PTEN* gene (Steck *et al*. 1997). PTEN's main mechanism of action is via its phosphatase activity in dephosphorylating the phosphoinositide phosphatidylinositol (3,4,5) trisphosphate (PIP_3) into phosphatidylinositol 4,5-bisphosphate (PIP_2) (Maehama and Dixon 1998) (Figure 1.2.1.1). This activity negatively regulates activation of RAC-alpha serine/threonine-protein kinase (Akt) (Stambolic *et al*. 1998; Myers *et al*. 1998). PTEN works in opposition to class I phosphatidylinositol- 3 kinase (PI3K), which phosphorylates phosphoinositides, increasing levels of PIP_3, which positively regulates Akt (Fruman *et al*. 1998; Denley *et al*. 2009; Vivanco and Sawyers 2002; Cantley and Neel 1999; Georgescu 2010). PTEN is known as a dual phosphatase, as it also has protein phosphatase activity which can dephosphorylate substrate proteins including Akt via direct interactions; both of these functions have a role in PTEN's tumour suppression activity (Furnari *et al*. 1998; Phadngam *et al*. 2016; Lee *et al*. 1999; Gildea *et al*. 2004). PTEN loss/mutation is found various forms of cancer cells including breast, thyroid, prostate

and glioblastoma, and even small alternations to the level of PTEN can encourage tumour growth (Alimonti *et al*. 2010; Morani *et al*. 2014; Steck *et al*. 1997; Ali *et al*.1999; Li *et al*. 1997). PTEN has additional important roles in neurons and plasticity; PTEN is reported to be essential in LTD, and is also involved in neuronal development and AMPAR trafficking (Moult *et al*. 2010; Jurado *et al*. 2010; Kwon *et al*. 2003).

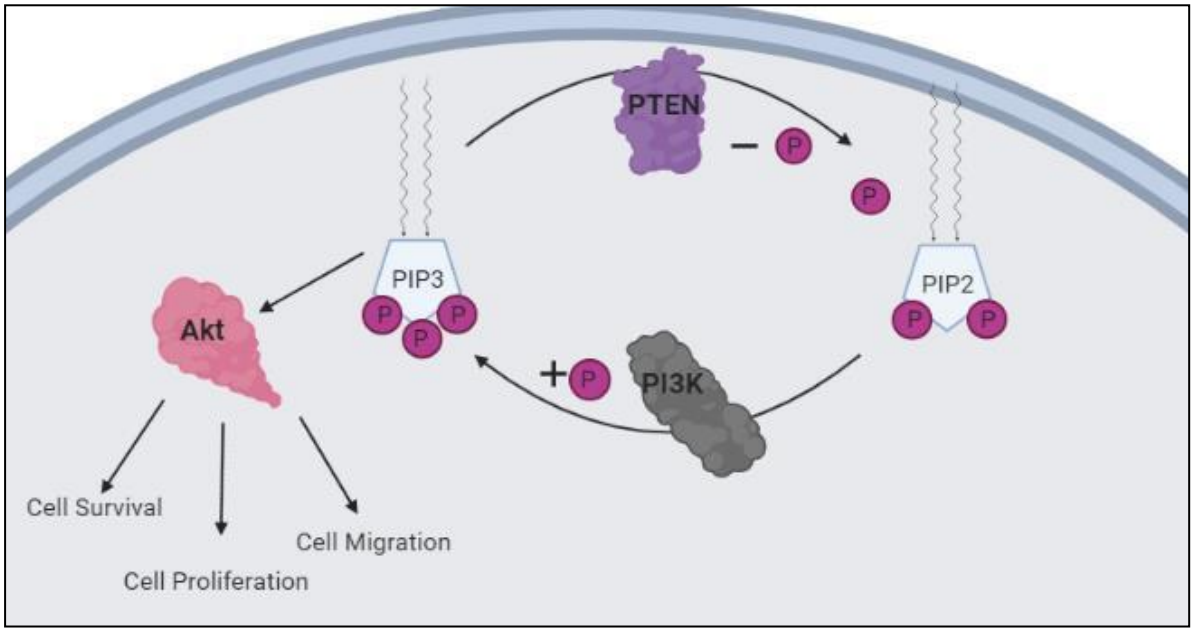

Figure 1.2.1.1. Schematic of PTEN/PI3K/AKT pathway.

PTEN dephosphorylates PIP_3, removing a D3 phosphate, recreating PIP_2. PI3K works in the opposite direction by adding a phosphate (this schematic is adapted from Sansal and Sellers (2004) and Carnero and Paramio (2014), and based on work by Maehama and Dixon (1998); Fruman *et al*. (1998), Denley *et al*. (2009); Vivanco and Sawyers *et al*. (2002); Cantley and Neel (1999); Georgescu (2010). Created on BioRender.com using premade shapes.

1.2.2 PTEN Signalling - Lipid Phosphatase Activity

PTEN's lipid phosphatase activity is critical in its role as a tumour suppressor; the dephosphorylation of PIP_3 limits activation of Akt, a major kinase which has over 100 known substrates including proteins involved in plasticity including Glycogen Synthase Kinase 3 (GSK3) and BCL2 associated agonist of cell death (BAD) (Manning and Cantley 2007; Cross *et al*. 1995; Datta *et al*. 1997). Akt has effects on many aspects of cellular homeostasis including cell survival, cell metabolism and protein synthesis, as well as synaptic plasticity (Myers *et al*. 1998; Manning and Cantley 2007; Datta *et al*. 1997; Levenga *et al*. 2017; Kohn *et al*. 1996; Stambolic *et al*. 1998). The absence of PTEN can lead to tumorigenesis through

lowered sensitivity to apoptosis and enhanced proliferation through the Akt pathway (Stambolic *et al*. 1998).

PIP_3 is rare and therefore very difficult to detect under unstimulated growth conditions, due to the necessary tight regulation of PI3K and activity of PTEN and other lipid phosphatases (Vivanco and Sawyers 2002). PIP_3 generation and subsequent Akt activation are strongly implicated in tumorigenesis (Stambolic *et al*. 1998; Miao *et al*. 2010; Denley *et al*. 2009), so it is not surprising that somatic and germline mutations in PTEN found in cancer mostly involve the phosphatase domain (Ali *et al*. 1999).

1.2.3 Phosphoinositide Activation of Akt

Although PIP_2 can bind Akt and partially activate it (Franke *et al*. 1997), PIP_3 binding has a stronger effect on activating Akt through allowing it to localise at the membrane, where it can then be phosphorylated at Thr308 by PIP_3-dependent protein kinase-1 (PDK1), in a PIP_3 Dependent reaction (Andjelkovic *et al*.1997; Stokoe *et al*. 1997; James *et al*. 1996; Alessi *et al*. 1997; Vivanco and Sawyers 2002). PIP_3 primes Akt for activation (Alessi *et al*. 1996; Andjelkovic *et al*. 1997). When PIP_3 binds Akt, it does not directly activate it, but causes its translocation from the cytosol to the membrane, and undergoes a change in conformation which allows PDK1 to activate it (James *et al*. 1996; Stokoe *et al*. 1997; Andjelkovic *et al*. 1997; Alessi *et al*. 1996; Milburn *et al*. 2003; Vivanco and Sawyers 2002). Although this phosphorylation of Akt at Thr308 is sufficient and critical to activate it, further activation can come from additional phosphorylation at Ser473 by PDK2 (Alessi *et al*. 1996; Alessi *et al*. 1997). Blocking PIP_3 binding to Akt using a nonphosphoinositide small molecule antagonist, which is able to block PIP_3–PH domain interactions (but not PIP_2 interactions), was able to supresses PI3K-PDK1- Akt pathway, and had downstream effects on inhibiting tumour growth and apoptosis (Miao *et al*. 2010). This shows PIP_3 binding to Akt can positively regulate it, and blocking this interaction can stop upstream kinases such as PDK1 from activating it (Miao *et al*. 2010).

1.2.4 PTEN Signalling - Protein Phosphatase Activity

Aside from lipid phosphatase activity, PTEN also has tyrosine, serine and threonine phosphatase ability (Myers *et al*. 1997). PTEN can directly dephosphorylate various substrate proteins including Akt, the GTPase Rab7, Focal Adhesion Kinase (FAK) and cAMP

Response Element-Binding Protein (CREB) (Tamura *et al*. 1998; Park *et al*. 2002; Gu *et al*. 2011; Phadngam *et al*. 2016; Shinde and Maddika 2016). This protein phosphatase activity plays a role in tumour suppression, as well as neuronal stem cell differentiation and gene expression (Tibarewal *et al*. 2012; Lyu *et al*. 2015). CREB phosphorylation is also relevant in synaptic plasticity, fear memory and adult hippocampal neurogenesis (Ortega-Martínez 2015; Zhou *et al*. 2009; Suzuki *et al*. 2011). Increasing CREB-mediated transcription can enhance short and long term memory storage via regulation of Brain-Derived Neurotrophic Factor (Suzuki *et al*. 2011).

In an ovarian cancer cell line (OVCAR3), PTEN-WT and PTEN with intact protein phosphatase (but not lipid phosphatase) activity can limit Glucose Transporter 1 (GluT1) surface expression and glucose uptake, which has a role in cancer (Zambrano *et al*. 2019; Phadngam *et al*. 2016). It is suggested that this is because PTEN physically interacts with Akt, and through its protein phosphatase activity can directly dephosphorylate it (Phadngam *et al*. 2016). Akt activation is known to regulate GluT1 expression (Barthel *et al*. 1999), so it was concluded that through protein phosphatase activity, PTEN can reduce Akt phosphorylation and have a downstream effect of limiting surface GluT1 expression (Phadngam *et al*. 2016). PTEN protein phosphatase activity can also dephosphorylate and subsequently deactivate FAK, reducing cell invasion in glioma cells (Tamura *et al*. 1998; Park *et al*. 2002). Recent work has also shown phosphoglycerate kinase 1 (PGK1), is a PTEN protein phosphatase target; its dephosphorylation by PTEN inhibits its autophosphorylation and subsequently activation (Qian *et al*. 2019). Through this pathway, PTEN is able to supress glycolysis and brain tumour formation (Qian *et al*. 2019).

1.2.5 PTEN Structure – N Terminus

PTEN's N terminus has a PIP_2 binding domain which is able to bind PIP and PIP_2; the binding of PIP_2 increases PTEN's phosphatase activity (Campbell, Liu, and Ross 2003; Liu *et al*. 2019). The N terminus is also involved in membrane anchoring; full length PTEN has a 30 times stronger affinity for artificial plasma membrane vesicles than the C2 domain alone, suggesting this area is critical for membrane localisation (Liu *et al*. 2019; Das, Dixon, and Cho 2003). The N terminus also contains lysine K13, which has been shown to be ubiquitinated, and is involved with nuclear localisation (Trotman *et al*. 2007; Liu *et al*. 2019). Next to this, a cytoplasmic localisation signal is also found (amino acids 19–25), mutations of which can also induce nuclear import (Denning *et al*. 2007). Mutation of this site can also reduce the ability of PTEN to supress growth and regulate phosphorylation of Akt at Ser473 (Denning *et al*. 2007).

1.2.6 PTEN Structure – Catalytic Domain

Next to the N terminus is the catalytic domain, with a large active site which can allow access for PIP_3 and other phosphorylated substrates; mutation of G129E in the site blocks PIP_3 access, but does not stop binding from protein substrates, meaning this PTEN "G" mutant has intact protein phosphatase activity, but no lipid phosphatase activity (Liu *et al*. 2019; Furnari *et al*. 1998). This mutation can inhibit PTEN's growth suppression activity, thus increasing cell proliferation (Furnari *et al*. 1998). The COOH terminal region forms a conserved domain (C2) in the C terminal which binds phospholipids (Lee *et al*. 1999) and is partially responsible for electrostatic membrane binding (Lee *et al*. 1999; Shenoy, Nanda, and Losche 2012; Das *et al*. 2003). The crystal structure shows the C2 domain contains a CRB3 loop which has five lysines with positive charge (Lee *et al*. 1999); Shenoy *et al*. (2012) have shown through MD simulations that this CRB3 loop is in contact with the membrane, and the C-tail is repelled away. Mutations in the CBR3 and Cα2 loops alter the tumour suppressor and membrane anchoring function of PTEN, without effecting its stability (Lee *et al*. 2019; Rahdar *et al*. 2009).

1.2.7 PTEN Structure – C-tail and PDZ Ligand

There are also four sites in the C-tail that can be phosphorylated: S380, T382, T383, and S385 (Das, Dixon, and Cho 2003; Rahdar *et al*. 2009; Vazquez *et al*. 2000) and this phosphorylation effects stability and activity (Vazquez *et al*. 2000). The relevance of these sites will be discussed in section 1.2.9.

At the end of PTEN then is a class I PDZ binding motif (Lee, Chen, and Pandolfi 2018). This sequence enables protein-protein interactions and regulates PTEN binding to proteins which contain a PDZ domain (Liu *et al*. 2019). Such proteins which can bind PTEN's PDZ bind motif include PSD-95 and MAGI2 (Jurado *et al*. 2010; Vazquez *et al*. 2001). PTEN-PDZ interactions are important in an array of processes in the cell including stability of PTEN and synaptic plasticity events in neurons (Valiente *et al*. 2005; Jurado *et al*. 2010). PDZ binding with MAGI-2 stabilises PTEN and PDZ binding with microtubule-associated serine/threonine (MAST) kinases enables phosphorylation of the C terminus of PTEN by these kinases (Valiente *et al*. 2005). PTEN mutants that had an intact PDZ domain, but mutations in the C terminal tail had differential PDZ binding with other proteins, showing it is not the PDZ binding motif alone that regulates PDZ binding; specific C terminal tail regions are also involved in PDZ domain recognition (Valiente *et al*. 2005). Acetylation of Lys402 regulates interactions between PTEN and PDZ domain-containing proteins (Ikenoue *et al*. 2008).

PTEN-PDZ association with PSD-95 is essential for LTD, as it helps PTEN to anchor in the presynaptic terminal (Jurado *et al*. 2010). PTEN's role in plasticity will be discussed in 1.2.14.

1.2.8 PTEN Localisation in Cell Lines

Localization of PTEN is important, as binding at the membrane is essential for its activity against PIP_3, and even low PTEN levels at the membrane can regulate PIP_3 levels (Nguyen *et al*. 2014; Vazquez *et al*. 2006). However, most PTEN is not membrane bound, it is in fact difficult to detect at the membrane without specialist microscopy (Nguyen *et al*. 2014; Vazquez *et al*. 2006). The process of PTEN membrane binding is highly dynamic, as PTEN only remains at the membrane for <400 ms (Vazquez *et al*. 2006). Because the cytosolic and membrane pools of PTEN change rapidly, there is no specific pool that is localised in one place, it is more likely that all PTEN is capable of membrane binding (Vazquez *et al*. 2006). Through molecular simulation data, it has also been shown that the mobility of nearby lipids is reduced in the presence of PTEN even if they don't directly interact with it, suggesting PTEN is able to regulate diffusive movement of lipids (Shenoy *et al*. 2012). Phosphoinositide binding events are lower than PIP_3 turnover rate, so it is suggested that each time PTEN binds lipids, there are several catalytic cycles (Vazquez *et al*. 2006).

As Yasui *et al*. (2014) note, several regions of PTEN are involved in membrane localisation and/or association with phospholipids including PIP_2 binding motif in the N-terminus, and this CRB3 loop and Cα2 helix in the C2 domain (Rahdar *et al*. 2009; Lee *et al*. 1999). The C2 domain binds phosphatidylserine in the CRB3 loop, helping to anchor at the membrane (Lee *et al*. 1999; Shenoy *et al*. 2012). Rahdar *et al*. (2009) confirmed PIP_2 binding is also critical in membrane binding; expression of a phosphatase specific to PIP_2 called inositol polyphosphate-5-phosphatase inhibited membrane binding. Non-specific electrostatic interactions at the membrane also enable membrane localisation (Das *et al*. 2003).

1.2.9 PTEN Conformation- Open/ Closed Model

PTEN has four phosphorylation sites (Ser380/ Thr382/ Thr383/ Ser385) which are close to each other on the C-terminal tail, and phosphorylation at these sites reduce its ability to localise at the plasma membrane and tapers its catalytic activity (see Figure 1.2.9.1. for schematic) (Das *et al*. 2003; Vazquez *et al*. 2000; Vazquez *et al*. 2001; Odriozola *et al*. 2007; Rahdar *et al*. 2009; Bolduc *et al*. 2013). It has been reported that phosphorylation at these sites causes PTEN to "switch" between an open or closed conformation (Odriozola *et al*. 2007; Vazquez *et al*. 2001).

Bolduc *et al*. (2013) made a tetra-phosphorylated PTEN mutant, at the four known phosphorylation sites in the C-tail. When compared to the unphosphorylated, PTEN-WT, this phospho-mimetic PTEN has a more compact conformation where the C-tail and C2 domain interact with each other. This phosphorylated, “closed” PTEN has reduced catalytic activity and membrane affinity, while dephosphorylated PTEN is in an “open” conformation, where its C-tail is not interacting with the C2 domain, leaving it able to associate with the membrane and carry out its lipid phosphatase activity (Bolduc *et al*. 2013; Rahdar *et al*. 2009; Das *et al*. 2003; Odriozola *et al*. 2007; Vazquez *et al*. 2000; Shenoy *et al*. 2012). Phospho-mimetic C-tail mutants are reported to have 80% lower affinity for membrane association (Das *et al*. 2003). Dephosphorylation triggers a conformational change, allowing nonspecific electrostatic interactions at the membrane; the association with the membrane is then strengthened by PIP_2 binding to the PIP_2 binding domain, and phosphoserine binding to the C2 domain (Das *et al*. 2003; Ross and Gericke 2009; Bolduc *et al*. 2013; Lee *et al*. 1999; Shenoy *et al*. 2012; Iijima *et al*. 2004).

The open/closed model was confirmed and expanded by Rahdar *et al*. (2009) N- and C-terminal fragments can be co-immunoprecipitated, whether expressed together or separately then mixed, demonstrating an intramolecular interaction. By mutating the phosphorylation sites in the C-terminal so that it was non-phosphorylatable, the PTEN fragments could then not associate, showing phosphorylation is necessary for this binding and the “closed” conformation (Rahdar *et al*. 2009). These mutations also increased membrane localisation. Mutation of the catalytic pocket or CRB3 loop also reduced intramolecular interactions; this site is where the C-tail is thought to interact (Rahdar *et al*. 2009). This provides further evidence of the intramolecular interactions of PTEN and how phosphorylation is involved. Furthermore, although the C-tail is needed to stabilise PTEN, without its C-tail can bind the membrane more easily and is actually more active, shown by its increase ability to facilitate G1 arrest (Rahdar *et al*. 2009; Vazquez *et al*. 2000). This confirms the notion that the C-terminal can have an inhibitory effect on membrane binding, as can phosphorylation of the C-tail.

There is some controversy surrounding exactly how PTEN switches conformation. As noted by Rahdar *et al*. (2009), they found an effect of mutation of charged residues K260, K263, K266, K267 and K269 in the CRB3 loop affected C-tail binding, while Odriozola (2007) did not see this effect. It is worth noting that the groups used slightly different constructs: Odriozola *et al*. (2007) used a full length PTEN and a fragment containing the C-tail, whereas

Rahdar *et al*. (2009) essentially cut PTEN in half. Both looked at how these pieces then associated with each other.

It has been reported that PTEN moves from the open to the closed conformation through a sequence of salt-bridge formations, where interactions move along the protein towards the C-terminal end; this happens extremely fast, within 100 nanoseconds (Shenoy, Nanda, and Losche 2012). Phosphorylated, closed PTEN is also less able to bind to PDZ-domain containing proteins such as MAGI-2, whereas in the “open” conformation it is more able to bind PDZ-domain proteins (Vazquez *et al*. 2001).

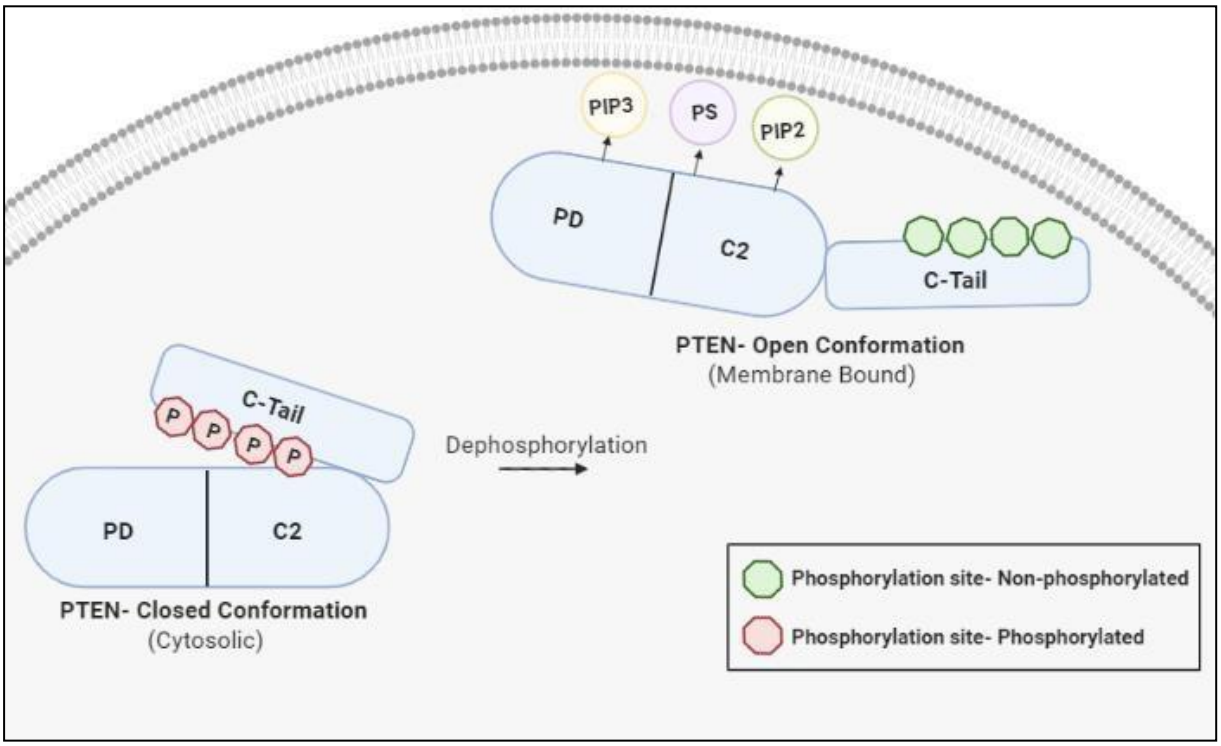

Figure 1.2.9.2. PTEN Open/ Closed Model.

When PTEN is phosphorylated at sites S380, T382, T383, and S385, it is in the closed conformation, with the C-tail wrapped around it, unable to bind the membrane (Das, Dixon, and Cho 2003; Rahdar *et al*. 2009; Vazquez *et al*. 2000; Bolduc *et al*. 2013). As explained by Ross and Gericke (2009), dephosphorylation causes its conformation to change and initially allows non-specific interactions at the membrane, this encourages the PIP_2 binding domain to bind PIP_2, and the C2 domain to bind phosphoserine, strengthening membrane association (Ross and Gericke 2009; Das *et al*. 2003; Lee *et al*. 1999; Iijima *et al*. 2004). This schematic from Ross and Gericke (2009) and based on work by Iijima *et al*. 2004; Lee *et al*. 1999; Redfern *et al*. 2008; Das *et al*. 2003; Rahdar *et al*. 2009; Vazquez *et al*. 2000).

1.2.10 PTEN Auto-Dephosphorylation

Raftapolou *et al*. (2004) discovered PTEN uses its own protein phosphatase ability to auto-dephosphorylate its T383 phosphorylation site in the C terminal, which is involved in enabling it to open and reveal its C2 domain. This enables the C2 domain to inhibit cell migration. Catalytically dead PTEN is more phosphorylated at T383 than PTEN-WT, so its own phosphatase activity plays a role in this regulation (Raftopoulou *et al*. 2004).

1.2.11 PTEN Orientation and Conformation

To further study the conformation of PTEN and compare the membrane bound and cytosolic differences in the protein, Shenoy *et al*. (2012) used neutron reflectometry and molecular dynamic simulations of PTEN; this technique can be used to analyse the composition of membranes, and conformation and orientation of proteins at the membrane (Wacklin 2010). When bound to the membrane, the structure of PTEN essentially flattened against the membrane (Shenoy *et al*. 2012). The C-terminal tail is in a different shape depending on whether PTEN is membrane bound or in solution; while PTEN is membrane bound it forms a peptide coil, which is stabilised by fluctuating hydrogen bonds, and its tail is repelled away from the membrane electrostatically by the membrane's acidic lipids (Shenoy *et al*. 2012). This enables PTEN to localise in the correct position at the membrane, with the lipid binding area towards the membrane, where phospholipids can anchor it in place (Shenoy *et al*. 2012). However, in solution, the C-terminal tail is wrapped around the C2 domain, which causes the end of the C-tail to block the membrane binding pocket (Shenoy *et al*. 2012). This study gives some insight into how the open/closed conformations are created, and why PTEN is inactive in its closed, non-membrane bound state and how PTEN positions at the membrane.

1.2.12 Summary- PTEN in Cell Lines

These studies together show that PTEN is a highly dynamic protein, which is regulated by phosphorylation, binding of phospholipids and phosphoserine and electrostatic interactions (Lee *et al*. 1999; Vazquez *et al*. 2000; Redfern *et al*. 2008; Das *et al*. 2003). Its localisation, conformation, interactions, stability and activity depend on these events, and this has downstream effects on cell cycle arrest, transcription and p-Akt signalling (Vazquez *et al*. 2001; Vazquez *et al*. 2000; Das *et al*. 2003; Bolduc *et al*. 2013; Rahdar *et al*. 2009). Higher levels of PTEN are membrane bound when cells are highly polarized after nutrient starvation, and membrane localisation may become asymmetrical (Vazquez *et al*. 2006). Therefore, the phenomenon of membrane association could be regulated in response to events in the cell,

to allow specificity during certain physiological situations, which may have an impact on tumour suppressor function (Vazquez *et al*. 2006). The ability of groups of PTEN molecules to be in this essentially inactive, cytosolic state allows for PI3K signalling and PTEN activity to balance PIP_3 levels (Vazquez *et al*. 2006).

1.2.13 PTEN in Neurons and Brain Development

PTEN is present in the pre and post-synapse in neurons (Weston *et al*. 2012; Takeuchi *et al*. 2013; Jurado *et al*. 2010). In hippocampal neurons, PTEN and phosphorylated PTEN localize at separate subcellular compartments; a T366 phosphomimetic mutant of PTEN is restricted to submembrane regions in the cytoplasm within the dendrites and cell body and mostly localised to the Golgi, whereas PTEN-WT localises more with synaptic and axonal markers and is more diffuse (Moult *et al*. 2010).

Some evidence of PTEN's importance in the brain comes from developmental studies. Neurons from conditional PTEN KO mice show enhanced neuronal projections and spine density, as well as defective synaptic structures in the cerebellum and cortex (Fraser *et al*. 2008). Myelination abnormalities were also present such as thickened axonal myelination, as well as weakening of synaptic transmission and plasticity in the hippocampus, compared to PTEN heterozygous or WT mice (Fraser *et al*. 2008). PTEN deletion also causes progressive macrocephaly in the mouse brain and increased phosphorylated Akt levels are observed (Kwon *et al*. 2003; Kwon *et al*. 2001). Mice with PTEN deletion also have neuronal hypertrophy with increased numbers of synapses, as well as ectopic dendrites in the cortex and hippocampus (Kwon *et al*. 2006). These mice displayed abnormal responses to stimuli and disrupted social learning and interaction. Furthermore, PTEN-deleted spines have a mushroom shape in comparison to spiny wild-types in granule neurons in the dentate gyrus, again showing a tendency towards enlargement during PTEN KO (Haws *et al*. 2014). These morphological differences are accompanied by increased Akt phosphorylation (Kwon *et al*. 2003), as well as changes to downstream Akt targets such as GSK3B (Kwon *et al*. 2006; Cross *et al*. 1995). These studies together show that PTEN has an important role in neuronal development in terms of regulating growth and synaptic transmission; and modulations to PTEN during development can have downstream effects on mouse behaviour (Kwon *et al*. 2003; Kwon *et al*. 2001; Fraser *et al*. 2008; Moult *et al*. 2010).

1.2.14 PTEN in Synaptic Plasticity- LTD

Despite being widely known for its role in tumour suppression, there is evidence that PTEN also has a critical role in synaptic plasticity (Jurado *et al*. 2010; Knafo *et al*. 2016). LTD is the

process by which after low frequency stimulation, synaptic responses are depressed (Lynch *et al*. 1977). It has been reported that PTEN is essential in this process (Wang *et al*. 2006; Jurado *et al*. 2010). When NMDA receptors are activated, PTEN is redistributed and recruited to the postsynaptic terminal where it binds PSD-95 (Jurado *et al*. 2010). When this mechanism is blocked in hippocampal slices using bpV(HO)pic, (a bis-peroxivanadium derivative and pharmacological inhibitor of PTEN), the magnitude of LTD involving AMPA receptors is greatly reduced in comparison to control slices, according to electrophysiological recordings (Jurado *et al*. 2010). Overexpression of a lipid and protein phosphatase-inactive PTEN mutant (PTEN-C124S) and a lipid phosphatase-inactive mutant which retains its protein phosphatase activity (PTEN-G129E) (Myers *et al*. 1998; Weng *et al*. 2001), both produced the same effects in blocking LTD; both mutants showed a reduction in sustained EPSCs up to 40 minutes after stimulation, suggesting the lipid phosphatase activity of PTEN is critical for its function in NMDAR-dependent, hippocampal LTD (Jurado *et al*. 2010). These manipulations of PTEN did not affect LTP, or hippocampal mGluR-dependent LTD in this study (Jurado *et al*. 2010).

Mechanistically, PTEN associates with PSD-95 through its PDZ ligand; Jurado *et al.* (2010) suggest that this interaction is critical in LTD, as expression of a PTEN mutant, which lacks the PDZ-binding motif (GFP-PTEN-ΔPDZ) blocks LTD (Jurado *et al*. 2010). Furthermore, overexpression of PTEN had an effect of depressing basal AMPAR transmission, but did not alter LTD. Interestingly, the amount of PSD-95 actually associated with PTEN under basal conditions is low at around 1%, which roughly doubles after NMDAR stimulation (Jurado *et al*. 2010). Therefore, even a low level of interactions of PTEN is significant in terms of plasticity events. Jurado *et al*. (2010) propose several potential mechanisms for how the PSD-95-PTEN interaction regulates plasticity. It could be that this interaction allows PTEN to localise by the membrane at activated synapses, which would improve its catalytic activity by allowing access to PIP_3, which is critical in AMPAR clustering at the membrane (Arendt *et al*. 2010). The group also suggest PTEN may regulate plasticity through GSK-3β, which is activated by PIP_3 (Cross *et al*. 1995) (See section 1.2.17).

This study suggests the phosphatase activity of PTEN, as well as its PDZ interaction ability is critical to NMDAR-Dependent LTD, and that PTEN expression can have a dampening effect on transmission (Jurado *et al*. 2010). Wang *et al*. (2006) also demonstrate the critical role of PTEN in LTD. They report that PTEN-deficient mice display LTP, but not LTD. It is suggested that this is due to the role of PTEN in PI3K suppression; PI3K pharmacological inhibition rescues LTD in PTEN-deficient mice. This study again highlights the importance of PTEN's phosphatase activity in LTD, but not LTP.

1.2.15 PIP_3 and Plasticity

Exactly how PTEN mechanistically regulates LTD is not clear, but due to the importance of its phosphatase activity (Jurado *et al*. 2010), it is possible that it may be through the regulation of PIP_3 (Arendt *et al*. 2010). Arendt *et al*. (2010) report that PIP_3 levels are implicated in the correct functioning of synaptic AMPARs. The exact location of AMPARs in the spine membrane is important, as around 15% of the spine surface area is considered the postsynaptic density (PSD), where neurotransmission occurs (Harris, Jensen, and Tsao 1992).

PIP_3 affects AMPAR distribution; depletion of PIP_3 causes an increase in the proportion of AMPARs that can cycle between the dendrite and spine (Arendt *et al*. 2010). This is because PIP_3 is necessary for PSD-95 localisation in spines; as PSD-95 is essential for AMPAR targeting to synapses, PIP_3 depletion causes AMPARs to detach from the PSD, and move away from this area, causing synaptic depression (Arendt *et al*. 2010; Chen *et al*. 2000). Inhibition of PIP_3 in neurons by quenching with Pleckstrin Homology domain from General Receptor for Phosphoinositides (PH-GRP1), which binds with PIP_3 with high affinity, causes depression of AMPAR-mediated EPSCs without effecting NMDAR EPSCs (Arendt *et al*. 2010; Corbin, Dirkx, and Falke 2004). The effect was then reversed when PIP_3 levels were restored by expression of a constitutively active PI3K.

Post-embedding immunogold electron microscopy was used in this study to establish precisely where AMPARs were localised (Arendt *et al*. 2010). It was reported that PIP_3 limits AMPAR surface expression; AMPARs localise to spines and accumulate at the plasma membrane during PIP_3 depletion (Arendt *et al*. 2010). In slices, PIP_3 synthesis inhibition with LY294002 caused GluA2 to localise in the extra-synaptic membrane next to the PSD, but GluA2 in the PSD was decreased (Arendt *et al*. 2010). Thus, PIP_3 levels control movement of AMPARs between the PSD and extra-synaptic compartment. Arendt *et al.* (2010) conclude that PIP_3 reduction causes local translocation of AMPARs in the spine surface, which leads to increased extra-synaptic AMPAR accumulation, and reduction of AMPARs at the synaptic membrane, which is congruent with depression of AMPAR-mediated transmission caused by PI3K inhibitor LY294002 or PH-GRP1 expression.

Mechanistically, PIP_3 may regulate the PSD-95 synaptic complex, which is critical for AMPAR clustering at the membrane (Arendt *et al*. 2010; Jurado 2017; Elias and Nicoll 2007; Bats *et al*. 2007). PIP_3 quenching through PH-GRP1 expression significantly decreased PSD-95 accumulation in spines (Arendt *et al*. 2010). Therefore, PIP_3 Dependent interactions may regulate translocation of PSD-95 to spines, and PIP_3 depletion may reduce the ability of

PSD-95 to anchor AMPARs at synapses, reducing clustering at the membrane (Arendt *et al*. 2010; Elias and Nicoll 2007; Bats *et al*. 2007).

This supports Jurado *et al*. (2010) who reported that suppression of PIP_3 levels via PTEN overexpression can reduce AMPAR synaptic responses, which they suggest may be due to reduced AMPAR clustering at the membrane. It is surprising that PTEN phosphatase inhibition can block LTD; (Jurado *et al*. (2010) give two potential explanations for this. They suggest that either overexpressed PTEN may act on a separate population of AMPARs from the group that are removed during LTD, or, synaptic depression induced by PTEN overexpression is not sufficient to saturate LTD expression (Jurado *et al*. 2010).

In support of Arendt *et al*. (2010), other work has demonstrated the involvement of PIP_3 and kinases in the PIP_3 pathway in AMPAR regulation; PI3K inhibition can reduce AMPAR membrane insertion and block LTP (Man *et al*. 2003; Jurado 2017). PIP_3 depletion could influence plasticity through inhibition of GSK3β (see 1.2.17) (Peineau *et al*. 2007; Jurado 2017; Cross *et al*. 1995). Additionally, PIP_3 may regulate AMPAR trafficking by driving AMPAR phosphorylation (Qin *et al*. 2005). GluA1 phosphorylation at S831 can occur via Ras–Pi3K–Akt pathway signalling, which drives its insertion into synapses (Qin *et al*. 2005). PIP_3 levels can also regulate the localisation of a variety of PIP_3-sensing proteins involved in AMPAR localisation including Sorting Nexin 27 (SNX27), Pleckstrin Homology Like Domain Family B Member 2 (PHLDB2) (McMillan *et al*. 2020; Cai *et al*. 2011; Hussain *et al*. 2014; Levi *et al*. 1993; Xie *et al*. 2019) (See General Discussion).

1.2.16 Evidence of PTEN Influence in Plasticity through PIP_3 Regulation

Following their work on PIP_3, Arendt *et al.* (2014) directly showed that PTEN counteracts PIP_3 signalling and shifts plasticity events towards depression. Under basal conditions, PIP_3 is being rapidly turned over in spines. NMDAR-Dependent LTP and LTD both upregulate PIP_3 levels, and during LTP, Akt phosphorylation at S473 and T308 was also increased (Arendt *et al*. 2014). PTEN is able to counteract PIP_3 increase during LTD, blocking the net change in PIP_3 (Arendt *et al*. 2014). Pharmacological inhibition of PTEN with bpV(HOpic) during LTD induction enhances PIP_3 levels in spines and the dendritic shaft compared to baseline (in addition to the initial increase seen after PTEN inhibition) (Arendt *et al*. 2014). In contrast, PTEN inhibition does not alter changes in PIP_3 levels seen after LTP. PTEN therefore functions as switch during plasticity which determines effects of PIP_3 upregulation, with a preference for LTD (Arendt *et al*. 2014). This study confirms the involvement of PTEN in plasticity through regulation of PIP_3 signalling, and again suggests PTEN has a preference

towards synaptic depression, an effect seen in other studies (Jurado *et al*. 2010; Knafo *et al*. 2016).

1.2.17 Plasticity Regulation through PTEN and GSK-3β

GSK-3β has a critical role in plasticity; conditional KO in mice leads to synaptic transmission defects, reduced NMDAR and AMPAR subunit and PSD-95 levels and impaired fear memory (Liu *et al*. 2018). Jurado *et al*. (2017) postulate that PTEN may enable LTD through its action of positively regulating GSK-3β. GSK-3β is reported to be activated during LTD; this activation is critical for LTD as GSK-3β inhibition blocks LTD (Peineau *et al*. 2007). GSK-3β is negatively regulated by AKT (Cross *et al*. 1995). Therefore, by reducing PIP_3 levels and subsequent Akt activation via its lipid phosphatase action (Maehama and Dixon 1998), PTEN can enhance GSK-3β activation (Sharma *et al*. 2002). This has been demonstrated in neurons; PTEN degradation is associated with increased GSK-3β phosphorylation (Kwak *et al*. 2010). It is suggested that during LTD, the translocation of PTEN to synapses has downstream effects through the PIP_3 pathway, with a result of activating GSK-3β at necessary synapses (Arendt *et al*. 2014; Jurado *et al*. 2010; Peineau *et al*. 2007).

1.2.18 PTEN in Synaptic Plasticity- LTP

While Jurado *et al*. (2010) have shown that PTEN is not a critical component in LTP, other groups have published contradictory results. Takeuchi *et al*. (2013) used theta burst-induced (TBS)-LTP a protocol of stimulation patterns involving short, high frequency bursts which can induce LTP in hippocampal neurons (Capocchi, Zampolini, and Larson 1992), to test the effect of PTEN KO on plasticity. The group found that in PTEN KO mice, TBS-LTP was dysregulated, this was in an age Dependent manner; TBS-LTP was increased in young KO mice and significant impairment was seen only when mice were middle-aged. In these middle-aged mice, synaptic transmission under basal conditions in dentate granule cells was enhanced (Takeuchi *et al*. 2013). There were also deficits in hippocampal-Dependent cognitive/social behaviour. As plasticity deficits are present at an earlier stage than morphological defects, it was suggested that PTEN KO effects on plasticity are not purely due to morphological defects (Takeuchi *et al*. 2013). Therefore, PTEN seems to have different roles/ relevance in different kinds of plasticity, possibly due to differences in methodology between studies (KO vs pharmacological inhibition and expression of PTEN mutants). PTEN has also been shown to robustly rescue synaptic deficits in Alzheimer's Disease models (Knafo *et al*. 2016) (see diseases Section 1.5).

1.2.19 PTEN and AMPAR trafficking

In addition to the mechanism discussed above, PTEN has been shown to regulate AMPAR trafficking via leptin, a hormone released from fat cells shown to be involved in NMDAR-dependent plasticity (Moult *et al*. 2010; Shanley, Irving, and Harvey 2001). Leptin has an inhibitory effect on PTEN though increasing its phosphorylation, and is also able to increase GluA1 surface expression in hippocampal neurons. This process also correlated with PIP_3 increase, and requires NMDAR activation (Moult *et al*. 2010). It was also shown that pharmacological PTEN inhibition with bisperoxovanadium (bpV) showed similar effects to those of leptin; GluA1 trafficking as well as excitatory synaptic strength were enhanced (Moult *et al*. 2010). When two dominant negative, catalytically dead forms of PTEN were expressed (G129E or C124S), basal mEPSCs amplitudes were increased and leptin application failed to produce further increases in EPSC's, further suggesting that a lack of PTEN activity influences basal EPSCs, and it is the phosphatase activity of PTEN that underlies this (Moult *et al*. 2010). This study suggests that inhibition of PTEN via leptin can enhance synaptic function in the hippocampus and influence AMPAR trafficking (Moult *et al*. 2010).

In summary, PTEN is an important regulator of PIP_3 in the context of plasticity, possibly through effects of PIP_3 on AMPAR trafficking and stability at the synapse, or through downstream effects on kinases such as GSK-3β (Arendt *et al*. 2010; Arendt *et al*. 2014; Jurado *et al*. 2010; Jurado 2017). The localisation of PTEN is relevant in this system, as PTEN is recruited to the post synaptic terminal during NMDA receptor stimulation, blocking its binding to PSD-95 reduces AMPAR mediated synaptic transmission (Jurado *et al*. 2010).

1.3 Post Translational Modifications

1.3.1 PTEN, Post Translational Modifications and Plasticity

PTEN can be modified by various Post Translational Modifications (PTMs) including SUMOylation, ubiquitination, phosphorylation and S-nitrosylation (Huang *et al*. 2012; Trotman *et al*. 2007; Kwak *et al*. 2010; Vazquez *et al*. 2000). Due to the relevance of both SUMOylation and ubiquitination in synaptic plasticity, and a role for PTEN (Jurado *et al*. 2010; Jaafari *et al*. 2013) this thesis aims to explore how these PTMs regulate PTEN and the relevance of this in plasticity, with a focus on protein trafficking (see Aims Section 2.). This is of interest given the evidence that S-nitrosylation and ubiquitination of PTEN have a role in AD pathology, and that modulation of PTEN activity can rescue Aβ related plasticity deficits

(Knafo *et al*. 2016; Kwak *et al*. 2010), PTMs relevant to PTEN and how they influence PTEN and plasticity will next be discussed.

1.3.2 SUMOylation

SUMOylation is the post-translational modification process whereby Small Ubiquitin-like Modifier (SUMO) protein is conjugated to a substrate (Matunis *et al*. 1996; Wilkinson and Henley 2010). SUMO can form covalent, reversible associations with substrate proteins, which can have a wide range of effects on cellular processes including inducing nuclear import of substrates, transcription, tumour necrosis factor-induced cell death and modulation of ubiquitin- mediated degradation (Shen *et al*. 1996; Matunis *et al*. 1996; Wang *et al*. 2014; Yang and Sharrocks 2004; Okura *et al*. 1996; Henley *et al*. 2020). SUMO was first identified in the 1990's and is critical for the function of eukaryotic cells (Wilkinson and Henley 2010; Shen *et al*. 1996; Matunis *et al*. 1996). SUMOylation is also important in many aspects of neuronal regulation (Henley *et al*. 2020).

Ran-GTPase-activating protein 1 (RanGAP1), a nucleotide guanosine triphosphate (GTP)ase which is involved in nuclear pore complex transport processes, was the first protein shown to be covalently modified by SUMO1 (Wilkinson and Henley 2010; Matunis, *et al.* 1996). It was shown that SUMOylation was able to influence target protein localisation: unmodified RanGAP1 localised in the cytoplasm, whereas SUMOylated RanGAP1 was enriched in the cytoplasmic fibres of the nuclear pore complex (Matunis *et al.* 1996; Mahajan *et al*. 1997). SUMO shares 18% of its sequence with ubiquitin, but differs in that it is not generally associated with subsequent degradation of substrates (Matunis *et al.* 1996; Matunis and Guzzo 2012; Gill 2004).

1.3.3 The SUMOylation Cycle

Before conjugation, the translated SUMO precursor must first be cleaved by SUMO specific proteases (SENPs), which reveals its carboxy-terminal Gly-Gly motif, enables adenylation by an E1 enzyme, enabling the formation of a E1SUMO thioester (Gareau and Lima 2010; Liebelt and Vertegaal 2016; Wilkinson and Henley 2010). SUMO is activated in an ATP-Dependent reaction, attaching it to the E1 enzyme, which is a heterodimer of SUMO-activating enzyme subunit 1 (SAE1) and SAE2; it is then transferred to an E2 enzyme (Ubc9), which enables conjugation to substrates by identifying SUMO consensus motifs (Desterro *et al*. 1999; Gong *et al*. 1999; Wilkinson and Henley 2010; Schwarz *et al*. 1998; Gareau and Lima 2010). Ubc9 facilitates the formation of an isopeptide bond between SUMO

and the substrate (Gupta *et al*. 2014). Around 75% of SUMO substrates contain consensus sequence “ψKX(D/E)”, where ψ is a hydrophobic residue, which can directly interact with Ubc9 (Gareau and Lima 2010; Xu *et al*. 2008; Gong *et al*. 1997; Sampson *et al.* 2001; Rodriguez, Dargemont, and Hay 2001). The ability of a substrate to interact with Ubc9 correlates with SUMOylation (Sampson *et al.* 2001). Ubc9 interactions with SUMO are much stronger than with ubiquitin (Gong *et al*. 1997).

E3 ligases, while not always essential for SUMOylation, can facilitate SUMO binding to substrates through assembling a complex consisting of an E2SUMO thioester with a substrate, or strengthening Ubc9 interactions with target proteins (Gareau and Lima 2010; Liebelt and Vertegaal 2016; Desterro *et al*. 1999). An example of an E3 ligase is Ran-binding protein 2 (RanBP2) which binds SUMO and Ubc9, and increases nuclear antigen SP100 SUMOylation (Pichler *et al*. 2002). In neurons, an E3 ligase called protein inhibitor of activated STAT (signal transducer and activator of transcription), PIASx, increases SUMOylation of transcription factor myocyte enhancer factor 2A (MEF2A) (Shalizi *et al*. 2007; Shalizi *et al*. 2006). Overexpression of PIASx enhances dendrite differentiation in the rat brain *in vivo*, which is suggested to be via increasing MEF2A SUMOylation (Shalizi *et al*. 2007). PIASx is therefore important for neuronal development and may be involved in neuronal connectivity (Shalizi *et al*. 2007).

SUMO can also bind non-covalently to substrates via SUMO-interacting motifs (SIMs), which are sites which vary in sequences, but often contain a hydrophobic core and aspartic flanking glutamic acid residues (Minty *et al*. 2000; Song *et al*. 2004; Kerscher 2007; Wilkinson and Henley 2010).

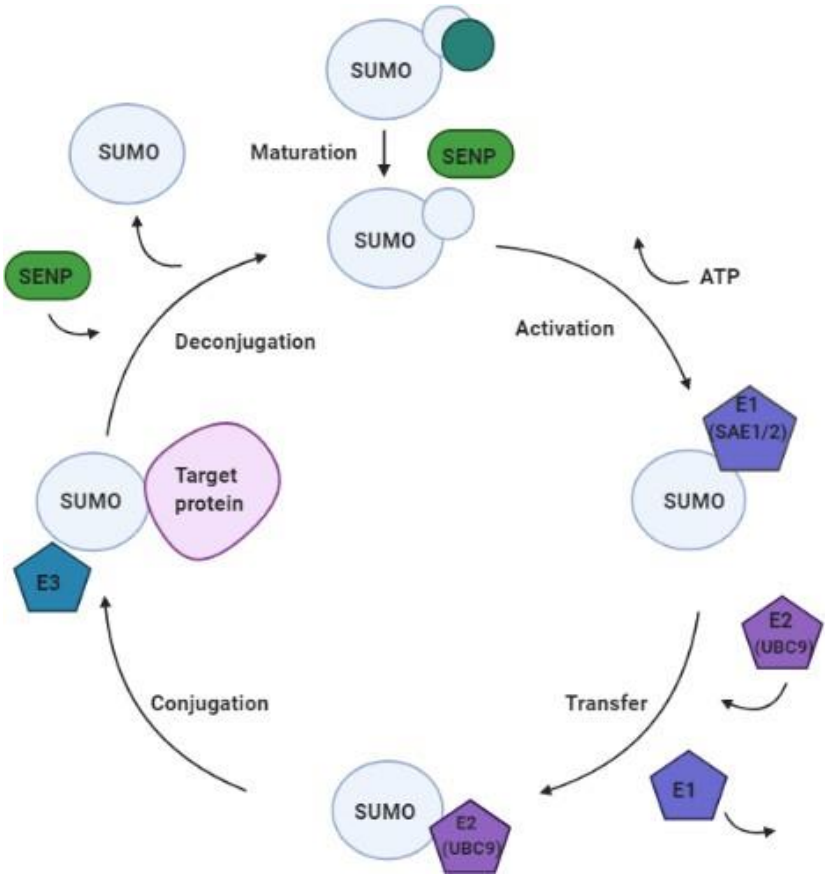

Figure 1.3.3.1. The SUMO cycle in mammals.

First, SUMO is cleaved by SENP, exposing its Gly-Gly motif, then activated in an ATP Dependent reaction, attaching it to the E1 activating enzyme (SAE1/2) (Guo and Henley 2014; Gareau and Lima 2010; Liebelt and Vertegaal 2016; Wilkinson and Henley 2010; Desterro *et al*. 1999; Gong *et al*. 1999). SUMO is then transferred to the E2 conjugating enzyme (Ubc9), which catalyses its conjugation to target proteins, in a reaction that is often facilitated by an E3 ligase (Gong *et al*. 1997; Gong *et al*. 1999; Wilkinson and Henley 2010; Schwarz *et al*. 1998; Gareau and Lima 2010). Finally, SENP can deconjugate SUMO from the target protein (Hickey, Wilson, and Hochstrasser 2012). This schematic is adapted from CytoskeletonNews, Xu *et al*. (2014), Meulmeester and Melchior (2008) and Chanda *et al.* (2018), and based on work by Desterro *et al*. (1999); Gong *et al*. (1999); Schwarz *et al*. (1998) and Sampson *et al*. (2001).

1.3.4 SUMO Paralogues and Chain Formation

One or more SUMO precursor proteins are found in all eukaryotes tested so far (Flotho and Melchior 2013). There are five paralogues of SUMO in mammals: SUMO1-5. SUMO2 is expressed in most tissues and ~46% identical to SUMO1 (Kamitani *et al*. 1998; Henley *et al*. 2020). SUMO 2 and 3 share the same sequence except for three N-terminal residues, so for simplicity, SUMO2 and SUMO3 with therefore be referred to as SUMO2/3 (Wilkinson and Henley 2010). Many target proteins can be SUMOylated by either isoform, while some are modified in a paralogue-specific manner (Wilkinson and Henley 2010). SUMO4 has a proline

residue which blocks its maturation and therefore cannot SUMOylate target proteins, but may be able to form non-covalent interactions (Owerbach *et al*. 2005).

An important quality of SUMO its ability to form chains, SUMO 2 and 3 contain an internal SUMOylatable lysine, meaning they can form chains through isopeptide bonds between this lysine on one molecule and the glycine residue on the C-terminal of another molecule (Vertegaal 2010; Xu, Plechanovova, *et al*. 2014; Tatham *et al*. 2001). SUMO1 lacks this site so it cannot form chains; it can however attach to SUMO 2/3 chains and act as a chain terminator (Vertegaal 2010; Matic *et al*. 2008; Tatham *et al*. 2001). SUMO 2 and 3 can also form polymeric chains with ubiquitin (Guzzo *et al*. 2012; Tatham *et al*. 2001).

1.3.5 DeSUMOylation

SUMOylation of substrates can be reversed by the same enzymes which cleave precursor SUMO, causing its maturation (Guo and Henley 2014; Wilkinson and Henley 2010). These enzymes are called SENPs, and can also cleave the isopeptide bond between the substrate and glycine of the attached SUMO, removing it from the substrate (Hickey, Wilson, and Hochstrasser 2012). SENPs fit into three categories as explained by Yeh (2009). The first contains SENP1 and 2, which are specific to mono-SUMO1, 2 and 3 (Guo and Henley 2014; Zhang, Saitoh, and Matunis 2002; Gong and Yeh 2006; Yeh 2009).

The next category includes SENP3 and 5, which preferential deSUMOylate mono-SUMO2/3, and to a lesser extent, SUMO1 (Guo and Henley 2014; Gong and Yeh 2006; Yeh 2009). SENP6 and 7 are in the final category and also prefer SUMO2/3; they preferentially deSUMOylate chains and have limited ability to cleave precursor SUMO (Guo and Henley 2014; Bekes *et al*. 2011; Mukhopadhyay *et al*. 2006; Yeh 2009). SENP cleavage of SUMO is reported to be rapid and stochastic, and blocking deSUMOylation results in slow growth and sensitizes cells to replication stress (Bekes *et al*. 2011).

1.3.6 Roles of SUMOylation in Non-Neuronal Cells

SUMO target proteins seem to be involved in nearly all aspects of cellular homeostasis and can be found widely in the cell, including on cell surface and mitochondrial proteins (Flotho and Melchior 2013; Zitti *et al*. 2017; Paasch *et al*. 2018). SUMOylation of substrates can have diverse effects, including modulation of DNA repair, antiviral response and ubiquitin-mediated degradation (González-Santamaría *et al*. 2012; Maccario *et al*. 2010; Huang *et al*. 2012; Huang *et al*. 2016; Wang *et al*. 2014; Bassi *et al*. 2013).

SUMOylation has also been shown to be critical in development; when SUMOylation is totally inhibited via KO of Ubc9 in mice, they die before E7.5 (Nacerddine *et al*. 2005). Cells deficient in Ubc9 also have severe deficits in nuclear organisation (Nacerddine *et al*. 2005). Specifically, SUMO1 absence is associated with cleft-pallet phenotype, although it is not essential for survival (Alkuraya *et al*. 2006), however, SUMO2 KO mice were not viable and died at E10.5 (Wang *et al*. 2014). SUMOylation also plays a role in ocular lens cell differentiation; SUMO1 binding to the transcription factor specificity protein 1 (Sp1) positively regulated it, increasing expression of β-crystallins (a marker of lens cell differentiation (Gong *et al*. 2014; Piatigorsky 1981). SUMO1 was present in complexes with Sp1 at both early and late developmental stages of mouse lens development (Gong *et al*. 2014).

SUMOylation is strongly implicated in the recruitment of proteasomal proteins to promyelocytic leukaemia nuclear bodies (PML-NBs); increasing accumulation of SUMOylated protein by using the proteasome inhibitor MG132 application induced 20S proteasome subunit translocation from the cytoplasm to the nucleus, where it showed higher levels of co-localisation with PMLs (Lamoliatte *et al*. 2017). Mutation of the SIM site in PML blocked this recruitment (Lamoliatte *et al*. 2017). Various proteins involved in SUMO regulation are also SUMOylated including Ubc9 and SAE1, as well as ubiquitin E3 ligases such as WWP2 (Lamoliatte *et al*. 2017; Bawa-Khalfe *et al*. 2017).

1.3.7 The SUMO Enigma

The SUMO system is highly dynamic; SUMO is continuously conjugated and deconjugated from substrates (Bekes *et al*. 2011), Due to the fact that generally there are low levels of SUMOylation of most substrates under basal conditions, but SUMOylation effects seem to persist after deconjugation (Wilkinson and Henley 2010), Hay (2005) suggests SUMOylation of a protein may have effects even after the SUMO has been deconjugated. This is referred to as the “SUMO enigma” (Hay 2005). For example, as explained by Henley and Wilkinson (2010), SUMOylation of kainite receptor subunit 6 (GluR6), induces its plasma membrane endocytosis (Martin *et al*. 2007). However, despite only a small fraction of GluR6 being SUMOylated under basal conditions, once endocytosed the subunit can be deSUMOylated and remain internalised – thus the effects of SUMOylation persist after its removal (Martin *et al*. 2007; Wilkinson and Henley 2010). This means that a previously SUMOylated protein may have a different localisation than a protein which has never been SUMOylated (Hay 2005; Wilkinson and Henley 2010).

1.3.8 Ubiquitination

Ubiquitin can covalently conjugate substrates and act as a signal for internalisation, proteasomal degradation or recruitment of other proteins, depending on which sites are ubiquitinated (Ciehanover, Hod, and Hershko 1978; Ciechanover *et al*. 1982; Varshavsky 2006; Komander 2009). There are three stages to ubiquitin conjugation. The first is carried out by an E1 enzyme which attaches to and activates ubiquitin, next the E1 ligase covalently links ubiquitin to an E2 ligase, then finally an E3 enzyme interacts with the E2 and substrate and is able to transfer ubiquitin to the substrate (Stewart *et al*. 2016; Suresh *et al*. 2016; Pickart and Eddins 2004). However, in some cases, E3 ligases can directly form a thioester with ubiquitin and transfer it to the substrate, without the need for an E2 ligase; one such E3 ligase is Homologous to E6AP C-Terminus (HECT) (Weber *et al.* 2019). HECT contains a catalytic cystine residue that can conjugate to ubiquitin (Weber *et al.* 2019). There are approximately 40 E2 enzymes in humans, but around 600–700 E3 ligases (Stewart *et al*. 2016; George *et al*. 2018).

Ubiquitin can conjugate to substrates in three formations: monoubiquitination, where one ubiquitin molecule binds a substrate, multi-monoubiquitination, where several molecules conjugate to a substrate at different sites, or polyubiquitination, where ubiquitin self-ubiquitinates on a substrate, forming chains (Komander 2009) (Figure 1.3.8.1.). Ubiquitin contains seven lysines which can accept ubiquitin which enable it to form chains (Komander 2009). Generally speaking, monoubiquitination of cell surface proteins leads to internalisation and lysosomal degradation or recycling, while polyubiquitin chains at Lys48 lead to proteasomal degradation (Kawadler and Yang 2006; Hershko and Ciechanover 1998; Haglund *et al*. 2003; Hicke and Riezman 1996; Komander 2009). Polyubiquitination at lys63 can induce signal cascade activation (Kawadler and Yang 2006). Alternatively, substrates can have their ubiquitin removed by deubiquitinating enzymes (DUBs), which deconjugate ubiquitin, which is then recycled through the ubiquitin-proteasome system (Suresh *et al*. 2016). Ubiquitin can form also mixed chains with SUMO (Nie and Boddy 2016), discussed further in Section 1.3.9.

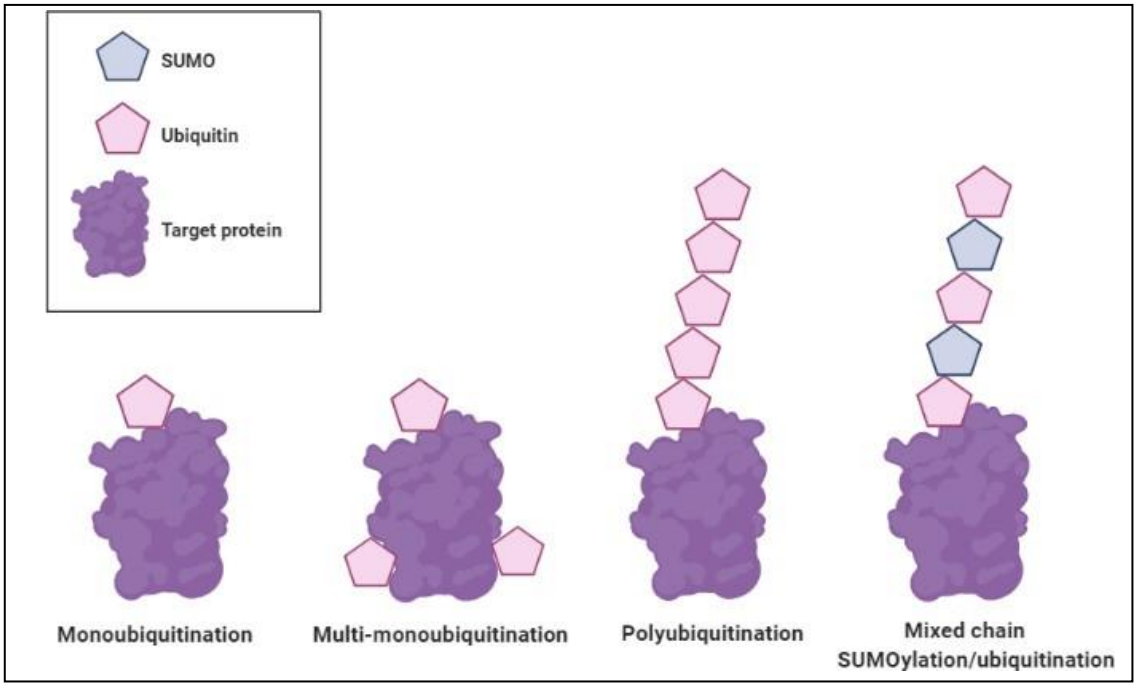

Figure 1.3.8.2. Ubiquitination Formations.

Ubiquitin can attach to target proteins in three formations; monoubiquitination, multi-monoubiquitination or polyubiquitination (Komander 2009). SUMO2/3 and ubiquitin can also form mixed chains on target proteins (Nie and Boddy 2016; Guzzo *et al*. 2012). This schematic is adapted from Sadowski and Sarcevic (2010) and Swatek and Komander (2016) and created in biorender.com using premade shapes.

1.3.9 Ubiquitination in Neurons and Plasticity

Ubiquitination has also been reported to be relevant in plasticity, including through regulation of AMPARs and AMPAR scaffolding proteins (Widagdo *et al*. 2015; Mabb and Ehlers 2010; Colledge *et al*. 2003). PSD-95 is ubiquitinated by E3 ubiquitin ligase Mouse Double Minute 2 Homolog (MDM2) (Colledge *et al*. 2003). This is triggered by NMDAR activation, and leads to PSD-95 degradation through the proteasome. By blocking PSD-95 ubiquitination through mutations of its PEST sequence, or treatment with proteasomal inhibitor MG132, NMDAR-induced AMPAR endocytosis is inhibited. LTD is also blocked by another proteasomal inhibitor Lactacystin, and significantly attenuated by MG132 (Colledge *et al*. 2003). This study highlights the role of ubiquitination in mediating PSD-95 regulation of AMPARs at synapses; PSD-95 ubiquitination via MDM2 is essential in regulation of AMPAR surface expression during plasticity (Colledge *et al*. 2003).

Lee *et al*. (2008) used contextual memory retrieval which involves conditioning mice with a fear stimulus, then re-exposure to induce fear memory, to examine ubiquitination of plasticity

proteins. After contextual memory retrieval, two PSD proteins, guanylate kinase associated protein (GKAP) and Shank are polyubiquitinated, and Shank is subsequently degraded (Lee *et al*. 2008). Mice fear-memory deficits caused by protein synthesis inhibitor anisomycin were rescued by proteasomal inhibition with clasto-lactacystin-β-lactone (βlac) in CA1 after retrieval. βlac also blocked the degradation of Shank after memory retrieval, keeping it up to basal levels. It was concluded that ubiquitin-Dependent degradation is part of the mechanism by which synapses are reorganised through protein degradation and synthesis in memory retrieval, and protein synthesis can rescue retrieval-induced degradation (Lee *et al*. 2008). Ubiquitination is therefore highly relevant to plasticity, through regulation of AMPAR endocytosis and synaptic reorganisation through protein degradation (Colledge *et al*. 2003; Lee *et al*. 2008).

1.3.10 SUMO and Ubiquitin Cross-regulation

There is a high level of overlap between proteins that are SUMOylated and ubiquitinated; many proteins are modified by both PTMs (Lamoliatte *et al*. 2017). SUMO and ubiquitin can also combine signals via a SUMO-targeted ubiquitin ligase (STUbL), which are ubiquitin E3 ligases which contains a SIM sites that allow it to bind SUMO chains and ubiquitinate them, forming mixed chains (Nie and Boddy 2016). An example is RING Finger Protein 4 (RNF4), which can tag SUMOylated proteins with ubiquitin (Nie and Boddy 2016; Xu, Plechanovova, *et al*. 2014). These mixed chains are recognised by proteins which have a SIM and ubiquitin interacting motif (UIM), which can have a range of effects including degradation, or recognition by proteins involved in DNA repair (Nie and Boddy 2016; Xu, Plechanovova, *et al*. 2014; Guzzo *et al*. 2012). The capping of chains by SUMO1 can increase binding to RNF4 (Xu, Plechanovova, *et al*. 2014).

1.3.11 Neuronal SUMOylation and Ubiquitination and Effects on Plasticity

SUMOylation of neuronal proteins can be increased by AMPA and potassium chloride stimulation (Feligioni *et al.* 2009). Increasing SUMOylation in the synapse is able to reduce potassium chloride-induced glutamate release, and reducing SUMOylation has the opposite effect (Feligioni *et al.* 2009). SENP1 increased potassium chloride-induced calcium influx and glutamate release. Interestingly, these effects were reversed when release was evoked with Kainate. It was concluded that SUMOylation of neuronal proteins can regulate neurotransmitter release (Feligioni *et al.* 2009).

At the post synapse, AMPARs are modulated by several kinds of PTMs including ubiquitination, phosphorylation and glycosylation (Diering and Huganir 2018; Widagdo *et al*. 2015). GluA1 and 2 are ubiquitinated near the end of their C-terminus when activated by AMPA, and this is critical for their ligand-induced degradation (Widagdo *et al*. 2015). This ubiquitination is necessary for AMPAR trafficking to late endosomes, where they undergo lysosomal degradation (Widagdo *et al*. 2015). Mutation of AMPAR ubiquitination sites (Lys868 in GluA1 and Lys870 or Lys882 in GluA2), reduced retrograde trafficking of the subunits to late endosomes and subsequently their degradation, without affecting surface levels (Widagdo *et al*. 2015).

Although AMPARs are not directly SUMOylated, their trafficking is regulated by global SUMOylation in neurons (Jaafari *et al*. 2013). This is required for insertion of GluA1 at the surface after glycine-induced LTP. This method of LTP induction also increases SUMO1 and Ubc9 mRNA in dendrites, as well as colocalization between SUMO1 and Ubc9 and PSD-95. SENP1 overexpression or dominant negative Ubc9 expression is able to inhibit LTP, shown by lack of increases in AMPAR surface expression, as well as lack of dendritic SUMO1 mRNA increase (Jaafari *et al*. 2013). It was shown that SUMO1 mRNA increases in spines are likely due to SUMO1 translocation, as mRNA binding protein: Cytoplasmic polyadenylation element binding protein (CPEB), was also increased after ChemLTP. This study suggests SUMOylation is critical for plasticity-mediated increases in AMPAR surface expression (Jaafari *et al*. 2013).

1.3.12 PTEN SUMOylation

SUMOylation of PTEN was first identified at two sites: K266 and K254 by Huang *et al.* (2012), who reported that K266R, K254R and K266R/K254R double PTEN point mutants are less SUMOylated than PTEN-WT in immunoprecipitation experiments. (See Figure 1.3.12.1. for schematic of PTEN SUMO sites). SUMOylation at K289 was also later discovered; in HEK cells, K266A mutation, and double mutation K266A/K289A either greatly reduced or almost abolished His-SUMO1 conjugation respectively compared to WT, and K266A and or K266A/K289A showed a similar effect for His-SUMO2 on greatly reducing conjugation (González-Santamaría *et al*. 2012). In that study, PTEN SUMOylation was examined in *in vitro* SUMOylation assays, where purified proteins are mixed together in a tube and immunoprecipitated. Wang *et al*. (2014) showed double mutant K266R/K254R had totally inhibited SUMOylation capacity, and single mutants were partially SUMOylated *in vitro* by SUMO1. They did not see a band shift between single and double mutants, which they suggest is because SUMO is large (>90 residues) so binding at one site blocks SUMOylation

at the other site. Huang *et al.* also saw this lack of band shift. Other than K254, K266 and K289 discussed, no other sites on PTEN have been proven to be SUMOylated, although SUMO Interacting Motif (SIM) sites have been found and non-covalent interactions between PTEN and SUMO1 are reported (Bawa-Khalfe *et al*. 2017). Bassi *et al.* (2013) report the dominant site to be K254, and (Wang *et al*. 2014) and Huang *et al.* (2012) showed double mutant K266R/K254R entirely blocks SUMOylation, however Gonzales-Santamaria *et al.* (2012) showed combined K266A/K289A mutation greatly reduced SUMOylation, so the dominant site cannot be unequivocally concluded.

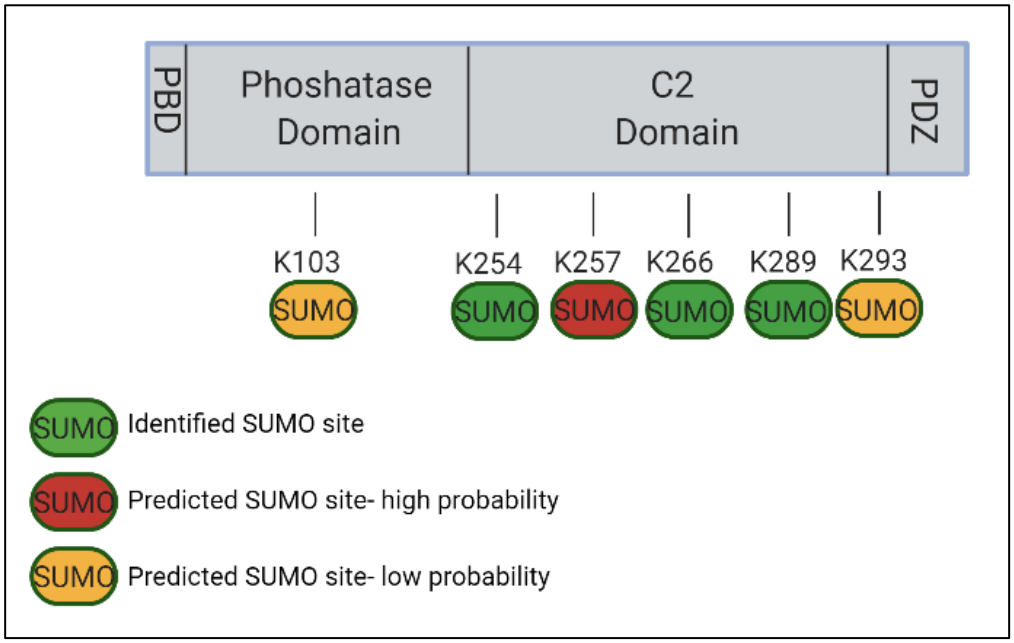

Figure 1.3.12.1. PTEN Predicted and Identified SUMO Sites. This schematic shows identified SUMO sites on PTEN in green (Huang *et al.* 2012; González-Santamaría *et al*. 2012). Predicted SUMO sites, according to SUMOplot software, are shown in yellow and red (sites with scores below 0.5 are omitted) (Abcepta, 2021). Created in Biorender.com using premade shapes.

An important point to note is that all studies except one on PTEN SUMOylation have tested the SUMOylation level of PTEN mutants using tagged, recombinant SUMO overexpression. An issue is that tagged proteins can behave differently to their endogenous counterparts (Skube *et al.* 2010), and overexpression of recombinant SUMO has been suggested to lead to artefacts if it is not controlled (Eifler and Vertegaal 2015). This will be discussed further in the general discussion. The only paper to test endogenous SUMO in cells did so by using SENP knockout cell lines (Bawa-Khalfe *et al*. 2017), which can confound results as it also causes changes to the global SUMOylation system. Therefore, although it is likely PTEN is SUMOylated, research in this area is still in the early stages and is perhaps confounded by overexpression and changes to global SUMOylation, especially taken with the lack of agreement in terms of which is the main SUMO site and how PTEN influences location, which will be discussed next.

1.3.13 Functional and Localisation Effects of PTEN SUMOylation - K254 Site

SUMOylation of PTEN has been found to have a range of effects on PTEN including effects on localisation, ubiquitination, antiviral response, DNA repair, ability to limit Akt pathway and tumorigenesis (González-Santamaría *et al*. 2012; Maccario *et al*. 2010; Bassi *et al*. 2013; Huang *et al*. 2012; Bawa-Khalfe *et al*. 2017). SUMOylation at K254, but not K289, was also found to enhance nuclear retention of PTEN, and is associated with response to genotoxic stress and DNA repair (Bassi *et al*. 2013). PTEN KD rescue with WT, but not lipid-phosphatase null or K254R mutants, was able to rescue response to ionizing radiation, shown by ability to recruit RAD51 to DNA damage sites which the mutants lacked. These mutants were also unable to resolve Breast cancer gene 1, early onset (BRCA1) expression compared to WT expressing cells (Bassi *et al*. 2013). K254R had equal phosphatase activity to WT in PTEN-deficient glioblastoma cell line U87MG (Bassi *et al*. 2013).

1.3.14 PTEN K266 SUMOylation Controls Membrane Binding and Tumour Suppression

Huang *et al.* (2012) propose that SUMOylation of PTEN at K266 facilitates membrane binding, and this is necessary for its phosphatase activity. They postulate that this happens due to an increase in electrostatic interactions between PTEN and the electronegative membrane, which are increased by the addition of SUMO, as molecular dynamics simulations showed it to have several positively charged sites (Huang *et al*. 2012). They report disruption of SUMOylation at this site reduce PTEN's ability to regulate the P-Akt pathway and downstream effects on tumour growth and cell proliferation. Reduced-SUMOylation capacity PTEN mutants (K266R and K254R mutants) had impaired ability to supress p-Akt pathway. In PC-3 cells, which contain a homozygous PTEN exon deletion, transfection with PTEN-WT reduced AKT phosphorylation at T308 and S473 by around 68% compared to control transfection cells (Vlietstra *et al*. 1998; Huang *et al*. 2012). In cells transfected with PTEN-K266R mutant, there was no significant change in Akt phosphorylation. In PTEN K254R mutant transfected cells, Akt phosphorylation was decreased by 38-39% compared to controls (Huang *et al*. 2012). When PC-3 cells expressing empty vector control or K266R PTEN were injected into immunodeficient mice, K266R mice had faster tumour growth than control mice, suggesting K266R mutation inhibits the tumour suppression ability of PTEN. Furthermore, Huang *et al.* (2012) saw a correlation between PTEN-SUMOylation-dependent Akt phosphorylation level and tumour growth. They also showed that K266 has a more cytosolic than membrane localisation compared to WT. Therefore, the integrity of K266 is necessary for PTEN's localisation and ability to regulate the Akt pathway. Blocking SUMO1 modification at these sites by mutating the lysine residues inhibits PTEN's ability to regulate the Akt pathway, thus promoting cell proliferation and tumorigenesis (Huang *et al*. 2012). Huang *et al.* (2012) postulate that SUMO modification of PTEN enables its activity independently of a conformational change, according to molecular dynamic simulations which were used to model the conformation of PTEN K266R, K266A and K266Q mutants.

Another study has reported the role of SUMOylation in PTEN membrane localisation (González-Santamaría *et al*. 2012). Imaging of MCF-7 cells showed PTEN mutants with reduced SUMOylation capacity (K266A, K289A and K266A/K289A mutants) localised predominantly in the cytoplasm, whereas PTEN-WT localised mainly in the nucleus. This effect was confirmed by subcellular fractionation and subsequent Western blot, which showed mutation at these sites alone or combined, switched PTEN localisation from nucleus to cytoplasm (membrane localisation was not tested at this point). Next, they showed that in

a model of viral infection, infection with Vesicular Stomatitis Virus induces PTEN translocation from cytosol to membrane, where it localises with SUMO1 and SUMO2 (González-Santamaría *et al*. 2012). Gonzales Santamaria *et al*. (2012) conclude that SUMOylation of lysines in the C2 domain of PTEN reduce the ability of the C2 domain to associate with C-terminus on PTEN, thus encouraging an open conformation, and increasing interaction with the plasma membrane. This differs from the view of Huang *et al*. (2012), who suggest SUMOylation effects PTEN independently of a conformation change. Conversely, Bawa-Khalfe *et al.* (2017) report that SUMOylated PTEN sequesters in the cytosol (although membrane localisation was not analysed specifically).

In summary, SUMOylation and PTEN localisation seem to be linked, although the mechanism by which SUMOylation facilitates membrane localisation is in dispute. The SUMOylation level of PTEN mutants has only been tested with endogenous SUMO in one paper, in which only SUMO1 modification (and not SUMO2/3) was detectable (Bawa-Khalfe *et al*. 2017). The role (or presence) of PTEN SUMOylation in neurons has not yet been examined; given the importance of both SUMOylation and PTEN in plasticity (Jurado *et al*. 2010; Jaafari *et al*. 2013), examining PTEN SUMOylation in neurons may further understanding of these processes.

Table 1. Literature Review of Previous Work on PTEN Mutants in SUMOylation Studies

Mutant	***Effect on SUMOylation***	***Effect on Location***	***Effect on Activity***	***Effect on Stability & Ubiquitination (Ub)***
K254R	Reduces SUMOylation (Huang *et al.* 2012; Bassi *et al.* 2013; Wang *et al.* 2014)	Inhibits nuclear retention (Bassi *et al.* 2013), Slightly reduces membrane binding (Huang *et al.* 2012)	Defective in P-Akt suppression (Huang *et al.* 2012)	
K266R	Reduces SUMOylation (Huang *et al.* 2012, Wang *et al.* 2014)	Inhibits membrane binding (Huang *et al.* 2012)	Can't supress P-Akt or tumour growth (Huang *et al.* 2012)	
K254R/ K266R	Reduces SUMOylation (Huang *et al.* 2012; Wang *et al.* 2014)			Increases Ub, reduces stability (Wang *et al.* 2014)
K266A	Reduces SUMOylation (Gonzales-Santamaria *et al.* 2012)	Increases cytoplasmic localisation (Gonzales-Santamaria *et al.* 2012)	Can't supress P-Akt (Huang *et al.* 2012)	Reduces Ub (Gonzales-Santamaria *et al.* 2012)
K266A/ K289A	Reduces SUMOylation (Gonzales-Santamaria *et al.* 2012)	Increases cytoplasmic localisation (Gonzales-Santamaria *et al.* 2012)		Reduces Ub (Gonzales-Santamaria *et al.* 2012)
K289A		Increases cytoplasmic localisation (Gonzales-Santamaria *et al.* 2012)		Reduces Ub (Gonzales-Santamaria *et al.* 2012)
K254/ K266A	Reduces SUMOylation (Bawa-Khalfe *et al*. 2017)	Promotes nuclear localisation (Bawa-Khalfe *et al*. 2017)		Increases stability (Bawa-Khalfe *et al*. 2017)

1.3.15 Ubiquitination of PTEN

Of the three SUMO sites mentioned (K266, K289 and K254), two are also known to be ubiquitination sites, K266 and K289 (González-Santamaría *et al*. 2012; Trotman *et al*. 2007; Wu *et al*. 2016). K13 and K66 are also reported to be ubiquitinated (Gupta and Leslie 2016; Trotman *et al*. 2007). K13 and K289 are functionally relevant as mutations at these sites inhibit nuclear import (Trotman *et al*. 2007). Importantly, K13 has been found to be

mutated in some forms of cancer (Duerr *et al*. 1998) and nuclear PTEN is reduced in late stage colon cancer (Trotman *et al*. 2007), K66 is thought to be the dominant ubiquitination site; combined K13R, K80R and K289R mutation reduced PTEN poly-ubiquitination levels somewhat compared to WT, but K66R alone greatly reduced FLAG ubiquitin immunoprecipitation by PTEN, suggesting this is the main site (Gupta and Leslie 2016).

1.3.16 Functional Consequences of PTEN Ubiquitination

Arguably, the most important consequence of PTEN ubiquitination is its subsequent degradation (Gupta and Leslie 2016). PTEN is downregulated in AD, and PTEN ubiquitination may regulate this PTEN loss (Griffin *et al*. 2005; Kwak *et al*. 2010) see 'Diseases' section for more information).

PTEN K66R mutation increases PTEN stability by reducing ubiquitination, which has downstream effects on reducing Akt activation and cell proliferation (Gupta and Leslie 2016). Trotman *et al.* (2007) reported that PTEN nuclear import is mediated by monoubiqutination, while degradation is mediated by polyubiquitination. Monoubiquitination-defective PTEN K289E mutant showed inhibited nuclear import; PTEN ubiquitination has important consequences as nuclear PTEN is more stable and there was a significant correlation between dominant nuclear (versus cytoplasmic) PTEN localization and low tumour stage in colon cancer (Trotman *et al*. 2007).

Maccario *et al.* (2010) also showed that PTEN's localisation at the membrane enhances its polyubiquitination, leading to destabilisation through enhanced degradation. Maccario *et al.* (2010) used N-terminal myristoylation of PTEN, which can be used to artificially induce membrane localisation (McIlhinney 1998). This artificially increased membrane targeting caused increased ubiquitination of PTEN (Maccario *et al*. 2010). This shows that the localisation of PTEN is also important in terms of regulating its level of ubiquitination and subsequent degradation.

Akt activation can also reduce PTEN stability through ubiquitination and degradation, which is dependent on the E3 ligase Makorin Ring Finger Protein 1 (MKRN1), which is stabilised through phosphorylation by Akt (Lee *et al*. 2015). It was concluded that there is a positive feedback loop whereby a reduction of PTEN activity increases Akt signalling, which promotes PTEN ubiquitination and degradation (Lee *et al*. 2015). Therefore, the relationship between PTEN, ubiquitination and PTEN activity is complex and bi-directional (Lee *et al*. 2015).

There is some debate about the role of PTEN ubiquitination on its catalytic activity. Akt phosphorylation suppression is reduced by mutation of K66R compared to WT, but this was

attributed to enhanced stability and subsequent increased levels of PTEN, rather than a change in its activity (Gupta and Leslie 2016). In contrast, Maccario *et al.* (2010) used a Ubiquitin “K all R” mutant, in which all seven lysines in ubiquitin are mutated, leaving it unable to form chains, to block poly-ubiquitination of PTEN (Li and Ye, 2008). This mutant blocked catalytical activity against PIP_3 (Maccario *et al*. 2010). It was concluded by Maccario *et al.* (2010) that ubiquitination reduces catalytic activity, and does so even before degradation, which is in contrast to Gupta and Leslie (2016) who suggest it is through enhanced degradation and subsequent reduced levels that catalytic activity is diminished.

1.3.17 SUMO/ Ubiquitin Crosstalk and PTEN

As described in Section 1.3.9, SUMO can recruit ubiquitin via a STUbL, forming mixed SUMO/Ubiquitin chains on a substrate which can act as a signal for degradation (Nie and Boddy, 2016; Xu *et al.* 2014). Bawa-Khalfe *et al.* (2016) demonstrated that SUMOylation of PTEN increases its association with WW Domain Containing E3 Ubiquitin Protein Ligase 2 (WWP2), an E3 ligase which facilitates PTEN ubiquitination and subsequent degradation. SENP1 deSUMOylates PTEN and WWP2, reducing WWP2-PTEN interaction and blocking ubiquitin mediated degradation (Bawa-Khalfe *et al.* 2016).

Conversely, SUMOylation can negatively regulate ubiquitination of PTEN (Wang, Chen, *et al*. 2014). Both SUMO1 overexpression, or overexpression of SUMO E3 ligase PIASxα reduced ubiquitination of PTEN, with a result of promoting PTEN stability. SiRNA KD of PIASxα had the opposite effect of reducing stability shown by reduced half-life of PTEN. Mutating two SUMO acceptor sites (K254 and K266) on PTEN, had an effect of ablating this PIASxα driven increase in stability through increasing ubiquitination (Wang *et al.* 2014). PIASxα also negatively regulated Akt phosphorylation by stabilising PTEN, and PIASxα overexpression induced cell cycle arrest, blocking proliferation and tumour suppression. Interestingly, in both PTEN null PC-3 cells and PTEN shRNA HeLa stable cell line, PIASxα overexpression did not have this effect of decreasing Akt phosphorylation, suggesting that this effect is PTEN Dependent. Furthermore, overexpression of PIASxα reduced the rate of cell proliferation in U2OS cells and HeLa cells, but there was no effect in PC-3 or shPTEN-HeLa stable cell line compared with control cells. It was concluded that PIASxα is a SUMO E3 ligase, and through its ability to increase PTEN SUMO1 modification, it can reduce PTEN ubiquitination and increase PTEN stability (Wang *et al.* 2014). This also suggests that SUMO and ubiquitin compete for the same sites on PTEN (Wang *et al.* 2014). Negative regulation of ubiquitination by SUMO1 has also been reported by Gonzales-Santamaria *et al.* (2012).

There is therefore conflicting evidence surrounding the order of SUMO and ubiquitin conjugation, and how they regulate each other.

1.4 The Retromer Complex

1.4.1 Retromer and PTEN Overview

A role for PTEN in the regulation of the trafficking complex “retromer” has recently been discovered (Shinde and Maddika, 2017). Retromer is important in neuronal function due to its ability to traffic neuronal proteins critical in synaptic plasticity such as AMPARs (Temkin *et al*. 2011; Temkin *et al*. 2017). Furthermore, there is evidence that the retromer pathway may be perturbed in AD and PD (for a detailed analysis see ‘Diseases’ Section 1.5) (Munsie *et al*. 2015; Mecozzi *et al*. 2014; Temkin *et al*. 2011). Therefore, due to the relevance of both PTEN and retromer in plasticity, and new evidence that PTEN can influence retromer (Jurado *et al.* 2010; Shinde and Maddika, 2017), examining how PTEN may influence retromer in cell models may give some insight into the development of synaptic pathology in neurodegeneration. As of yet, little is known about how PTEN influences retromer, and which cargoes are affected. PTMs of PTEN have also not been examined in the context of retromer trafficking. Retromer is involved in the sorting/trafficking of over 150 proteins including receptors, integral membrane proteins and ion channels (Cullen and Steinberg 2018; Steinberg *et al*. 2013; Steinberg *et al*. 2012; Fjorback *et al*. 2012). Such proteins include GluT1 and Alanine, Serine, Cysteine Transporter 2 (ASCT2) (Cai *et al*. 2011; Kvainickas *et al*. 2017). Retromer is also relevant in neurons and AD due to its ability to traffic Beta-Secretase 1 (BACE1), Beta-2 Adrenergic Receptors (β2ARs) and AMPARs and has a significant role in plasticity (Wang *et al*. 2012; Temkin *et al*. 2011; Choy *et al*. 2014; Mecozzi *et al*. 2014).

1.4.2 The Retromer Complex

Generally speaking, when membrane proteins are trafficked away from the membrane, they go through one of two pathways: after absorption by clathrin-coated vesicles and subsequent retrieval by early endosomes, they are either recycled back to the membrane, or remain in early endosomes which then mature into late endosomes, leading to degradation via the lysosomal pathway (Kornfeld and Mellman 1989; Mellman 1996; Huotari and Helenius 2011; Burd and Cullen 2014; Elkin *et al.* 2016). Most proteins are recycled back to the plasma membrane via early endosomes, only a small proportion are degraded (Huotari and Helenius 2011). An important part of this system is the retromer complex, which is mostly involved in regulating trafficking of various cargoes from endosomes back to the cell surface (the

recycling pathway) (Seaman *et al*. 1997; Vagnozzi and Pratico 2019). Deficits in retromer leads to mis-sorting of cargoes, and subsequent lysosomal degradation (Lucas *et al*. 2016). Retromer has been described as a "*master conductor of endosome sorting*" (Burd and Cullen 2014), and is conserved across all eukaryotes (Koumandou *et al*. 2011).

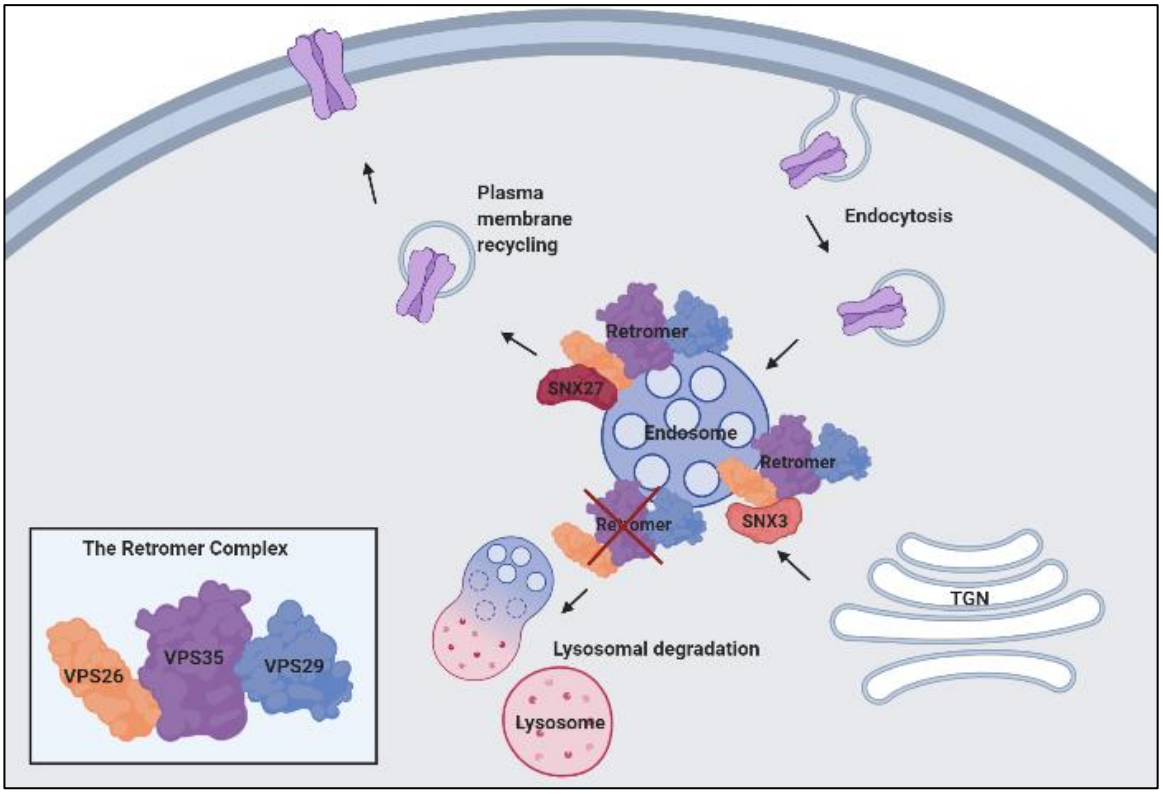

Figure 1.4.2.1. The Retromer Pathway.

After endocytosis, cell surface proteins are engulfed by early endosomes; they then undergo degradation through the lysosomal pathway, or are recognised and retrieved by retromer, which recycles them back to the plasma membrane (Wang *et al*. 2018; Gallon and Cullen 2015). For some proteins, SNX27 is also necessary for recycling back to the cell surface (Simonetti *et al*. 2019; Steinberg *et al*. 2013; Gallon *et al*. 2014; Yong *et al*. 2020; Rabouille 2017). This schematic is adapted from Yong *et al.* (2020), Lee *et al.* (2016), Wang *et al.* (2018) and Gallon and Cullen (2015) and Rabouille, (2017) and created in biorender.com with pre- made shapes.

1.4.3 Structure of the Retromer Complex

Retromer was initially discovered in yeast in the late 1990's, and includes proteins encoded vacuole protein sorting (*VPS*) genes (Seaman 2012; Seaman *et al*. 1997; Seaman *et al.* 1998). Mammalian retromer is not stable, it is created by the transient association of its components, including SNX proteins, and the VPS26:VPS29:VPS35 trimer, which recognises cargoes (Seaman *et al.* 1998; Swarbrick *et al*. 2011). The VPS trimer is

generally thought to be the main part responsible for cargo recognition, with VPS35 being the main component in this (Burd and Cullen 2014). Most cargoes contain the following hydrophobic motif necessary for sorting: “F/W-L-M/V,” (Seaman, 2007; Burd and Cullen 2014). VPS26 can also recognise cargo and direct cargo localisation; KD of VPS26 lead to a more peripheral re-localization of Sortilin Related Receptor 1 (sorLA), it is thought that VPS26 facilitates retrieval of sorLA from endosomes to the Golgi (Fjorback *et al.* 2012). A highly conserved N-terminal motif in VPS35 (PRLYL), is responsible for interactions with VPS26, and VPS26 can regulate VPS35 membrane association (Gokool *et al*. 2007). VPS26 can also regulate interactions between VPS35 and sorting nexins (Reddy and Seaman 2001).

1.4.4 Sorting Nexins

Sorting nexins (SNX proteins), are associated with the retromer complex which are involved in trafficking away from degradative pathways (van Kerkhof *et al*. 2005). Of the 33 identified in mammals, only six are thought to interact with retromer: SNX1, SNX2, SNX3, SNX5, SNX6 and SNX27 (Lucas and Hierro 2017). SNX proteins contain a phox (PX) domain; this is a membrane interacting domain which can bind phosphoinositides (Chandra *et al*. 2019). The PX domain characterises SNX proteins and allows PIP_3 binding (Seet and Hong 2006), enabling them to localise at endosomes where PIP_3 is enriched (Carlton *et al*. 2004; Cheever *et al*. 2001; Worby and Dixon 2002; van Kerkhof *et al*. 2005; Fields *et al*. 2010; Lucas and Hierro 2017; Cai *et al*. 2011). SNX proteins then have different roles depending on the rest of their structure; broadly speaking, as explained by Lucas *et al*. (2016), SNX proteins fit into three groups: those which only possess a PX domain, those which also contain a BAR domain and SNX27, which is the only SNX to contain a FERM and PDZ domain, and can regulate potassium channel expression (Wassmer *et al*. 2007; Peter *et al*. 2004; Lunn *et al*. 2007; Temkin *et al*. 2011; Steinberg *et al*. 2013; Burd and Cullen 2014; Balana *et al*. 2011; Clairfeuille *et al*. 2016; Ghai *et al*. 2013). SNX27 is known as an adaptor protein; adaptor proteins can recognise sorting motifs and mediate interactions between cargoes and retromer (Burd and Cullen 2014). SNX27 is enriched in early endosomes and localises there through interactions with PIP_3 (Cai *et al.* 2011). SNX27 knockout in mice leads to enhanced NMDAR expression and growth retardation, they also die within 3 weeks of birth (Cai *et al.* 2011). It was concluded that SNX27 is important in development and may mediate endocytosis or endosomal sorting (Cai *et al.* 2011). SNX27 is able to regulate sorting of cargo from recycling through its ability to association with retromer; it can link proteins to

retromer via its PDZ ligand and is therefore known as an adaptor protein (Temkin *et al*. 2011; Gallon *et al*. 2014; Steinberg *et al*. 2013; Damseh *et al*. 2015; McMillan *et al*. 2020). However, SNX27 is not always necessary for retromer-mediated trafficking, in some cases, the VPS trimer is able to recycle proteins without SNX27 (Steinberg *et al*. 2013). An analysis of over 100 cell surface proteins has found that under VPS35 KD conditions, over 50 proteins were reduced at the cell surface which were not further reduced by SNX27 KD (Steinberg *et al*. 2013). GTPases Rab7a and Rab5 are necessary for VPS35/29/26 complex recruitment to membranes (Seaman *et al*. 2009; Rojas *et al*. 2008).

While not retromer associated, SNX17 is important for maintaining the stability of membrane proteins and regulating their distribution; disruption of the SNX17 binding region of low-density lipoprotein receptor-related protein (LRP) results in reduced LRP degradation and expression at the membrane, suggesting SNX17 is important in recycling (van Kerkhof *et al*. 2005). SNX17 is also able to regulate recycling of integrins and promote their stability through its FERM-like domain, without the recruitment of retromer (Steinberg *et al*. 2012). SNX3 has been shown to be involved in endosome to Golgi transport in a retromer Dependent pathway (Cui *et al*. 2019).

1.4.5 The Retromer Component VPS35 in Neurons and Plasticity

Retromer is involved in trafficking of many neuronal proteins including β2 adrenergic receptors (β2ARs), AMPARs, NMDARs and dopamine transporters (Munsie *et al.* 2014; Choy *et al.* 2014; Wu *et al.* 2017; Cai *et al.* 2011). Retromer protein VPS35 is important in AMPAR trafficking and function (Tian *et al*. 2015; Temkin *et al*. 2017). In PSD and synaptosomal samples from neurons from VPS35-depleted transgenic mouse brains, total GluA1 and GluA2 levels were also significantly decreased, and there were less mature spines (Tian *et al*. 2015). GluA2 overexpression could rescue this spine maturation deficit. Mini EPSCs were also lower compared to readings from neurons from WT mice, and AMPAR subunits were reduced at the surface (Tian *et al*. 2015).

VPS35 is also reported to be critical in LTP; VPS35 KD blocked LTP in mouse hippocampal slices and neuronal cultures, while LTD was unaffected (Temkin *et al.* 2017). Spine density of dendrites in CA1 pyramidal cells were reduced in VPS35-KD cells. In imaging experiments in neurons, VPS35 KD induced AMPAR translocation from early endosomes, suggesting retromer is involved with sorting of AMPARs, possibly by ensuring they are sorted into vesicles and available to be inserted into the membrane during LTP (Temkin *et al.* 2017). It was concluded that VPS35 is important in trafficking of AMPARs in plasticity (Temkin *et al.* 2017), which is supported by evidence that VPS35 KD reduces total AMPAR levels (Binda *et*

al. 2019). Basal AMPAR transmission was not affected by VPS35 KD (in contrast to Choy *et al*. (2014) who found an effect under basal conditions).

Retromer also plays a developmental role in neurons; VPS35 is involved in dendritic growth; Reducing VPS35 in mouse embryonic hippocampal neurons leads to fewer spines, shorter dendrites and swollen axons (Wang *et al*. 2012). This suggests VPS35 KD causes deficits in protein trafficking in developing neurons in mice (Wang *et al*. 2012).

1.4.6 Retromer Component SNX27 in Neurons and Plasticity

SNX27 is also critical to neuronal function; SNX27 KO in mice leads to growth retardation in developing mice, and these mice die within three weeks (Cai *et al.* 2011). The PDZ binding motif of NMDAR receptor 2C (NR2C) is recognised by the SNX27 PDZ domain, and SNX27 localises on early endosomes through its PX domain interaction with PIP_3 (Cai *et al.* 2011). SNX27 KO in neurons leads to enhanced NR2C levels and aberrant NR2C endocytosis; suggesting that SNX27 can regulate the endosomal sorting or endocytosis of NR2C (Cai *et al.* 2011).

SNX27 is also involved in regulating AMPAR receptor trafficking (McMillan *et al*. 2020), and there is evidence that SNX27 can directly interact with AMPARs (Hussain *et al*. 2014). However, some reports suggest that rather than binding AMPARs directly, SNX27 binds leucine-rich repeat and fibronectin type-III domain containing protein 2 (LRFN2), which is involved in AMPAR trafficking (McMillan *et al*. 2020).

This study involved injection of shRNA-expressing lentivirus into rat brains to knock down SNX27 or LRFN2; 6-8 weeks later hippocampal slices were made from these rats. SNX27 KD profoundly reduced field EPSCs, and LFRN2 KD also caused a significant reduction (McMillan *et al*.2020). LFRN2 KD also blocked LTP induction. In rat cortical neurons, SNX27 KD reduced GluA2 surface expression by 34%, while LRFN2 KD caused a 40% reduction. SNX27 KD also leads to a 50% reduction of total LRFN2. LRFN2 was suggested by McMillan *et al.* (2020) to act as link between SNX27 and AMPARs, which regulates their surface expression and transmission. The group also concluded that SNX27 is required for maintaining levels of LRFN2 expression, and subsequent LRFN2 reduction after SNX27 depletion leads to reduction of surface GluA2 expression and defective transmission (McMillan *et al*. 2020). Other reports have shown that SNX27 overexpression can upregulate surface GluA1 and NMDAR subunit NR1, and SNX27 KD has the opposite effect (Wang *et al*. 2013). These studies together provide evidence that retromer proteins such as VPS35 and SNX27 are involved various processes relevant to neuronal development and function

including plasticity, neurotransmission and receptor trafficking (Temkin *et al.* 2017; McmilIlan *et al.* 2020; Cai *et al.* 2011).

1.4.7 PTEN Can Influence Retromer Through Direct Interactions

Recent research has found a direct link between PTEN and retromer (Shinde and Maddika *et al.* 2017). PTEN can directly associate with SNX27, blocking its association with VPS26 and sequestering it away from retromer. This reduces the ability of retromer to traffic GluT1 from the endosome to the membrane of HepG2 and HeLa cells, and limits glucose transporter GluT1 expression at the surface (Shinde and Maddika, 2017). PTEN shRNA KD leads to increased surface GluT1 and glucose uptake levels compared to control shRNA cells. The authors discovered the T401I mutation in the PTEN PDZ binding motif is not able to obscure SNX27-VPS26 as the wildtype does, which also increases surface GluT1 compared to expression of PTEN-WT, suggesting PTEN has an important role in regulating surface GluT1 levels and the PDZ binding motif of PTEN is critical in this. PTEN binds SNX27 next to its VPS26 binding site; Shinde and Maddika (2017) explain that this can obstruct SNX27-VPS26 binding and is able to reroute GluT1 away from Rab11-positive recycling endosomes, towards lysosomes shown by co-localisation with LAMP1, leading to degradation. This may influence tumour progression due to a downstream effect on glucose uptake. Influence of PTEN phosphatase activity and through the Akt pathway in this context were ruled out, as a catalytically dead PTEN mutant did not associate with SNX27 differently to WT, and pharmacological Akt inhibition also did not alter SNX27-PTEN or SNX27-VPS26 interaction (Shinde and Maddika, 2017).

1.5 PTEN, PTMs and Retromer in Disease Pathology

1.5.1 Overview

Although PTEN is primarily known for its tumour suppression function, it also plays key roles in synaptic plasticity (Jurado *et al*. 2010; Liu *et al*. 2018). Intriguingly, this may be relevant to Alzheimer's disease (AD), as PTEN inhibition is reported to relieve AD-related plasticity deficits, and PTEN is dysregulated in AD brains (Griffin *et al*. 2005; Knafo *et al*. 2016). AD, which accounts for 70% of dementia cases, is generally associated with synaptic dysfunction and enhancement of LTD (Kametani and Hasegawa 2018; Ondrejcak *et al*. 2010). PTEN has a preference for synaptic depression in neurons, and can enhance AD-related synaptic depression (Knafo *et al*. 2016; Arendt *et al*. 2014). PTEN is therefore a potential therapeutic target in AD, as suppression of PTEN activity can rescue AD-related synaptic dysfunction and learning and memory deficits in mouse models (Knafo *et al*. 2016).

The retromer complex is also critical in plasticity through its ability to regulate trafficking of neuronal proteins such as AMPARs, and is therefore is also associated with neurodegeneration (Temkin *et al*. 2017; Munsie *et al*. 2014). PTEN may influence trafficking of AMPARs, and has recently been shown to influence retromer-mediated trafficking (Moult *et al*. 2010; Shinde and Madikka, 2017). Examining the influence of PTEN on retromer mediated trafficking in cell models may therefore provide a useful avenue to further understand the role of PTEN in plasticity and neurodegeneration.

PTMs are also a point of interest in this context, as SUMOylation is implicated in plasticity and AD, as well as cancer (Xie *et al*. 2014; Hung *et al*. 2019; Jaafari *et al*. 2013; Lee *et al*. 2014), and there is evidence that SUMOylation, ubiquitination and phosphorylation are important regulators of PTEN function (Huang *et al*. 2012; Rahdar *et al*. 2009; Trotman *et al*. 2007). Ubiquitination may play a role in PTEN loss seen in AD brains (Kwak *et al*. 2010; Griffin *et al*. 2005), highlighting the relevance of PTMs and PTEN in neuronal function and neurodegeneration. The role of PTEN-SUMOylation in plasticity has not yet been examined. It is also not yet known whether PTMs of PTEN are relevant to its role in retromer mediated trafficking. This section will outline disorders associated with PTEN, retromer and PTMs, with a focus on neurodegeneration.

1.5.2 Alzheimer's Disease- Amyloid- Beta

Alzheimer's Disease is partially characterised by the accumulation of plaques of highly insoluble peptides such as Amyloid beta (Aβ) (Selkoe 1991; Murphy and LeVine 2010). Build-up of these peptides in neurons is suggested to begin the pathogenesis of Alzheimer's disease (Selkoe 1991; Glenner and Wong 1984; Chen *et al*. 2017), and is created via proteolytic processing of amyloid precursor protein (APP), by β- or γ-secretases, such as beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) or Presenilin-1 (PS1) (Cai *et al*. 2001; Bustos *et al*. 2017; Chow *et al*. 2010; Chen *et al*. 2017). Cleavage of APP by secretases is not very precise, which results in variation between Aβ species, but around 5-10% is $A\beta_{42}$, which is thought to be more fibrillogenic than other Aβ species and the cause of most deposits (Murphy and LeVine 2010). BACE1 is the main protease which generates $A\beta_{42}$ through APP cleavage (Cai *et al*. 2001). APP is cleaved by these proteases under normal conditions, but during AD, Aβ is accumulated to a greater extent due to a lack of degradation, leading to plaques and neurotoxicity (Hardy and Higgins 1992; Kametani and Hasegawa 2018; Selkoe 1991; Murphy and LeVine 2010).

Generally speaking, $A\beta_{42}$ is associated with LTP inhibition and LTD enhancement (Ondrejcak *et al*. 2010). Application of $A\beta_{42}$ oligomers to hippocampal slices at concentrations as low as 200pM can influence neurotransmission; this was reported to increase mini EPSC frequency and reduce paired pulse facilitation (Gulisano *et al*. 2019). Changes were associated with a higher number of docked vesicles at the presynapse and longer postsynaptic density length, suggesting $A\beta_{42}$ oligomers influence neurotransmitter release and postsynaptic structure (Gulisano *et al*. 2019). This study gives some insight into the mechanism by which $A\beta_{42}$ exerts its effects.

There is evidence that Aβ can contribute to pathogenesis through effects on AMPARs (Chang *et al*. 2006). Chang *et al*. (2006) found AMPAR mediated synaptic scaling contributes to AD related pathology in double knock in (2KI) (APP/Presenilin-1) mutant mice. These mice have mutations in APP: K670N/M671L (mutated human Aβ sequence) and presenilin-1: P264L, causing them to have increased $A\beta_{42}$ levels from 6 months of age as well as more plaques, without APP overexpression (Flood *et al*. 2002; Chang *et al*. 2006). These 2KI mice showed age-related downregulation of AMPAR-mediated basal and evoked currents and synaptic AMPAR decrease in CA1 cells. The mice had age-related deficits in both LTD and LTP, and impaired memory flexibility (Chang *et al*. 2006). This study shows through reducing AMPARs at the synapse and reducing AMPAR transmission, $A\beta_{42}$ may give rise to synaptic deficits and cognitive impairment (Chang *et al*. 2006).

Although it seemingly contrasts with Gulisano *et al*. (2019) who saw a $A\beta_{42}$-related increase in neurotransmission, these differences are consistent with the glutamate excitotoxic theory of AD, which explains that excessive or prolonged glutamate signalling may be neurotoxic (Doble 1999). AD could be due to Aβ-mediated changes that could cause neurodegeneration as the diseases progresses, after memory impairment (Esposito *et al*. 2013). Considering this theory, it follows that the initial increase in transmission seen by Gulisano *et al*. (2019) after acute Aβ treatment could lead to neurodegeneration through excitotoxity, which could present as age-related AMPAR downregulation seen by Chang *et al*. (2006) in aging model mice. It is also of note that there was variation in methodology between these studies (transgenic mice vs $A\beta_{42}$ application, and differences in current recording protocols).

1.5.3 Alzheimer's Disease – Tau

AD is also characterised by the accumulation of abnormal fibres in neurons called neurofibrillary tangles (NFTs), which are made up of highly insoluble peptides such as Tau (Kametani and Hasegawa 2018; Braak *et al*. 1986; Pirscoveanu *et al*. 2017). Tau was discovered in 1975 as a Microtubule-Associated Protein (MAP), which helps to assemble microtubules, and could be immunoprecipitated with Tubulin (Weingarten *et al*. 1975). Tau pathology is thought to be one of the main causes of various neurodegenerative diseases including AD and Parkinson's Disease (PD) (Braak *et al*. 1986; Pirscoveanu *et al*. 2017; Gendron and Petrucelli 2009). In healthy conditions, Tau is generally localised in axons (Binder, Frankfurter, and Rebhun 1985); in "tauopathies", Tau forms neuritic plaques, neurofibrillary tangles (NFTs) and neuropil threads, often containing paired helical filaments (PHFs) (Braak *et al*. 1986; Pirscoveanu *et al*. 2017). There are varying phenotypes between the different diseases in terms of the localisation and characteristics of Tau pathologies, but one particular marker is usually present; NFTs formed of hyperphosphorylated, insoluble Tau in paired helical filament form or twisted filaments (Braak *et al*. 1986; Gendron and Petrucelli 2009). Tau hyperphosphorylation depresses biological activity of healthy Tau, and seems to precede the assembly of tau filaments (Pirscoveanu *et al*. 2017).

1.5.4 Alzheimer's Disease and Excitotoxic Glutamate Signalling

It is suggested that AD can be the result of glutamate toxicity, and dysregulation of glutamate signalling may be involved in learning and memory deficits in AD (Greenamyre and Young 1998; Wang and Reddy 2017). Glutamate must be tightly controlled, as excess release can cause hyperexcitability of neurons; this prolonged activation can eventually be excitotoxic and lead to cell death of the post synaptic cell (Doble 1999; Hollmann and Heinemann 1994;

Wang and Reddy 2017). This excitotoxicity is thought to arise from the neuronal swelling that occurs after calcium influx, and lack of desensitisation of AMPARs after stimulation can also lead to cell death as this is protective against excessive stimulation by glutamate (Koh and Choi 1991; Koh *et al*. 1990; Jensen *et al.* 1998). Excessive AMPAR activation and calcium conductance through Calcium-Permeable (CP) AMPARs can lead to induced neuronal death (Mahajan and Ziff 2007). Mechanistically, uninhibited stimulation of AMPARs causes membrane depolarization and NMDAR activation (Hollmann and Heinemann 1994). This is because after glutamate or AMPA-induced stimulation, AMPARs rapidly undergo desensitisation after ligand binding, which involves a change in conformation of the dimer interface, which closes the channel and reduces conductance, inactivating the receptors (Sun *et al*. 2002). In AD, excessive NMDAR and AMPAR stimulation leads to calcium dysregulation and neurotoxicity (Armada-Moreira *et al*. 2020; Alberdi *et al*. 2010). Aβ oligomers significantly increase calcium currents through NMDARs and AMPARs, leading to oxidative stress and mitochondrial dysfunction in entorhinal–hippocampal slices and cortical neurons, and cell death in hippocampal slices (Alberdi *et al*. 2010). Furthermore, Aβ is also reported to potentiate potassium-evoked calcium currents in cortical and hippocampal slices at nanomolar concentrations (Kabogo *et al*. 2010). There are therefore several ways in which Aβ can cause synaptic dysregulation, including through increasing glutamate release and calcium influx through AMAPARs and NMDARs (Alberdi *et al*. 2010; Kabogo *et al*. 2010).

1.5.5 PTEN and Alzheimer's Disease Pathology

PTEN is dysregulated in AD brains; total PTEN levels are reduced in the entorhinal cortex, while PTEN levels are increased in CA1 dendrites (Griffin *et al*. 2005). Furthermore, Akt levels are reduced, while phospho-Akt is increased in AD brains compared to controls. Importantly, neurodegeneration and plasticity-related Akt targets GSK and Tau were also more phosphorylated (Griffin *et al*. 2005; Jiang *et al*. 2005; Peineau *et al*. 2007; Gendron and Petrucelli 2009). PTEN loss, and Akt and GSK activation were correlated with Aβ plaque deposition (Griffin *et al*. 2005). This study suggests that PTEN and the Akt pathway may be dysregulated in AD (Griffin *et al*. 2005), and other work has supported the finding that PTEN is dysregulated in AD brains (Kwak *et al*. 2010). PTEN has also been implicated in AD related plasticity deficits in mouse models (Knafo *et al*. 2016). Knafo *et al*. (2016) found that Aβ induced synaptic depression, and PTEN overexpression also mimicked and occluded this in neuronal cultures and mouse models. Aβ induced PTEN translocation into the postsynaptic compartment, which is a PDZ-dependent event, and this mechanism is critical for Aβ-induced synaptic depression. Treatment with $A\beta_{42}$ induced NMDAR-Dependent LTD in hippocampal slices from WT mice, but not from knock in mice with PTEN lacking the PDZ mutant. Treatment with a PTEN-PDZ-interaction blocking peptide, was also able to rescue

synaptic depression after Aβ treatment. This study suggests PTEN may be the link between Aβ and Alzheimer's related synaptic depression, and highlights the importance of PDZ interactions of PTEN in Aβ-related neuropathology (Knafo *et al*. 2016). It is also congruent with Griffin *et al*. (2005), who showed that PTEN levels are enhanced in CA1 dendrites in AD brains, again suggesting the role of PTEN in neurodegeneration. NMDAR stimulation causes PTEN accumulation in the PSD, which is critical in LTD (Jurado *et al*. 2010). Knafo *et al*. (2016) therefore suggest the Aβ -driven accumulation of PTEN in the synaptic compartment may therefore enhance PTEN-mediated synaptic depression, which may occur though removal of AMPARs due to changes in PIP_3 levels (Arendt *et al*. 2010). According to Knafo *et al*. (2016), this link between Aβ, PTEN and AD suggests that Aβ can hijack and enhance PTEN-mediated mechanisms of synaptic depression, which plays a role in AD pathology.

1.5.6 Ubiquitination of PTEN and Alzheimer's Disease

As mentioned, SUMOylation and ubiquitination have a role in plasticity (Lee *et al*. 2014; Colledge *et al*. 2003). PTEN is critical in LTD, plays a role in Aβ-induced pathology and is dysregulated in AD brains (Jurado *et al*. 2010; Griffin *et al*. 2005; Knafo *et al*. 2016). Due to the importance of PTMs in the regulation of PTEN (Huang *et al*. 2012), ubiquitination and S-nitrosylation of PTEN have been tested in the context of neurodegeneration (Kwak *et al*. 2010). The brains of people with the early stages of Alzheimer's disease showed reduced total PTEN levels and enhanced P-Akt compared to controls, as well as enhanced S- nitrosylation of PTEN (Kwak *et al*. 2010). It was shown that increased S-nitrosylation of PTEN in cortical rat neurons, caused by treatment with Nitric Oxide (NO) donor S-nitroso cysteine (SNOC), leads to reduced PTEN levels through enhanced E3 ubiquitin-protein ligase NEDD4-1 mediated ubiquitination and subsequent degradation (Kwak *et al*. 2010). A triple PTEN mutant (C71/C83/C124A) was unable to be nitrosylated and this mutant also displayed less ubiquitination that WT, which Kwak *et al*. (2010) suggest may be ubiquitination of PTEN is in part Dependent on PTEN nitrosylation. SNOC treatment also inhibited PTEN's lipid phosphatase activity, evidenced by increases in PIP_3 after SNOC treatment in a malachite green assay (an *in vitro* enzyme assay using bacterially expressed, purified PTEN). Interestingly, treatment of cultured neurons with glutamate or Aβ also increased PTEN S- nitrosylation and ubiquitination, as well as increased Akt phosphorylation (Kwak *et al*. 2010). Therefore, PTEN stability and phosphatase activity can be regulated by S-nitrosylation and ubiquitination, which may explain the PTEN loss seen in Alzheimer's disease, and provides a link between PTEN PTMs and effects of Aβ (Griffin *et al*. 2005).

1.5.7 Neuronal SUMOylation and Alzheimer's Disease

SUMOylation in neurons can influence plasticity (Jafaari *et al*. 2013), and is implicated in Alzheimer's disease (Lee *et al*. 2014; Qin *et al*. 2019). SUMOylation dysregulation is found in Tg2576 AD mouse models, as well as post-mortem AD brains (Lee *et al*. 2014). Tg2576 mice shown predominantly SUMO2/3 dysregulation, and minimal SUMO1 dysregulation. Lee *et al*. (2014) found slices from Tg2576 mice, and WT slices incubated with $A\beta_{42}$ oligomers both showed LTP inhibition. $A\beta_{42}$ oligomer application inhibits the natural increase in SUMOylation found after neuronal stimulation with KCI, which they suggested is the cause of LTP inhibition (Lee *et al.* 2014). Expression of dominant negative Ubc9 (DN-Ubc9) can also impair this stimulation-induced SUMOylation and reduces LTP (Lee *et al*. 2014). Furthermore, in CA1 pyramidal neurons, expression of SENP1, completely blocked single-cell LTP, again highlighting the role of SUMOylation.

This plasticity inhibition from SUMOylation depletion was reflected in behavioural experiments; infusion of DN-Ubc9 20 minutes before or after foot shock training significantly impaired freezing behaviour the day in a contextual fear conditioning (FC) task and performance in the Morris water maze test; it was found DN-Ubc9 infusion after training sessions impaired performances in later sessions (Lee *et al*. 2014). Ubc9 perfusion was also able to rescue deficits in behavioural test scores after $A\beta_{42}$ oligomer perfusion (Lee *et al.* 2014). Expression of Ubc9 could also rescue LTP deficits both in slices from Tg2576 mice, and slices from WT mice after $A\beta_{42}$ oligomer application, evidenced by increased fESPC slope in CA1 after theta burst stimulation. These experiments together show that enhancement of SUMOylation can significantly improve plasticity deficits and cognitive function in chronic Aβ-induced pathology; and hippocampal-dependent reference memory influenced by SUMOylation (Lee *et al*. 2014).

More recently, SUMOylation of Protein phosphatase 2 (PP2) has been found to contribute to AD pathology (Qin *et al*. 2019). PP2 is a serine/threonine phosphatase with a wide range of targets in the cell, and represents a collection of enzymes with the same catalytic subunit rather than a single enzyme (Mumby 2007). PP2A inhibition induces tau hyperphosphorylation, a main contributor of NFTs (Liu *et al*. 2008; Qin *et al*. 2019). PP2 is regulated by an endogenous PP2 inhibitor called SET (Qin *et al*. 2019). Qin *et al*. (2019) report that SET can be SUMOylated by SUMO1 *in vitro* and *in vivo* at K68, and SET was more SUMOylated in mice with three AD-related mutations: PS1m146v, APPswe and TauP301L, compared to control mice.

Qin *et al*. (2019) also found that SUMOylation of SET causes its cytoplasmic retention and PP2A inhibition, with a downstream effect of tau hyperphosphorylation. Non-SUMOylated SET showed preferential localisation in the nucleus. SET SUMOylation also induced cognitive defects in mice including long term memory; expression of WT, but not non-SUMOylatable SET mutant K68R via injection into CA1 in mice, caused reduced freezing times in contextual fear tests. Expression of WT SET was associated with reduced levels of PSD-95, GluA1, synaptotagmin and NMDAR subunit NR2A compared to vector expressing group (Qin *et al*. 2019). Furthermore, expression of non-SUMOylatable SET mutant K68R rescued expression of these proteins to a level similar to the control group. These results suggest SET-SUMOylation limits expression of synaptic proteins involved in plasticity, which modulates its effect on memory defects (Qin *et al*. 2019). Furthermore, Aβ oligomer treatment increased SET SUMOylation in rat hippocampal neurons. It was concluded that SUMOylation of SET enhances its retention in the cytoplasm, which blocks PP2A activity leading to Tau hyperphosphorylation, and that through SET SUMOylation, Aβ can enhance neurodegeneration (Qin *et al*. 2019).

SUMOylation therefore has a range of targets in neurons, and is able to influence plasticity and related processes including neuronal protein expression and neurotransmission (Lee *et al*. 2014; Qin *et al*. 2019). This has effects on long term memory and learning, and is influenced by Aβ (Lee *et al*. 2014; Qin *et al*. 2019).

1.5.8 PTEN in Learning and Memory- PTSD

In an innovative study by Lui *et al*. (2018), repetitive transcranial magnetic stimulation (rTMS), a kind of whole brain stimulation, was shown to relieve Post-Traumatic Stress Disorder (PTSD)- associated plasticity deficits through inhibition of the PTEN/Akt pathway. A mouse model of PTSD was induced using single prolonged stress (SPS) treatment on mice, which caused synaptic plasticity impairment, increased PTEN levels, reduced number and length of dendrites. It also lowered levels of the following NMDAR subunits: NR1, NR2A, NR2B and AMPAR subunits: GluA1, GluA2, GluA3 and GluA4 in the anterior cingulate cortex (ACC) (Lui *et al*. 2018). RTMS was able to significantly rescue the reduced glutamatergic receptor expression and decrease PTEN levels (Lui *et al*. 2018). The SPS-induced reduction of number and length of dendrites was also reversed by rTMS, and significantly enhanced by inhibition of PTEN using siRNA PTEN or PTEN inhibitor Bispovanadium (BpV). SPS also induced Akt dephosphorylation, which was blocked by BpV and PTEN siRNA. Blotting and confocal microscopy revealed HrTMS reversed the SPS-induced decrease of P-AKT, an effect enhanced further by siRNA PTEN or BpV. This is explained by the ability of HrTMS to

partially block SPS-induced increase in PTEN expression (Liu *et al*. 2018). The effect of rTMS to block the reduction of NR2B and GluA1 induced by SPS, was also significantly increased by addition of siRNA PTEN or BpV. Effects through the Akt pathway were further confirmed by use of Akt inhibitor Wortmannin in conjunction with rTMS. Wortmannin inhibited the effects of rTMS on Akt phosphorylation, and simultaneously lowered GluA1 and NR2B expression, again suggesting the involvement of the PI3K/Akt pathway (Liu *et al*. 2018). This study provides useful evidence that at the whole animal level, PTEN is highly involved in the context of PTSD mouse models, and has its effects through the Akt pathway by influencing glutamate receptor expression and synaptic morphology.

1.5.9 PTEN in Neurodegeneration- SMA and Axonal Regeneration

In addition to AD and PTSD, there is evidence that PTEN attenuation can have a neuroprotective effect in other neuropathology including Spinal muscular atrophy (SMA) and may enhance cell survival through effects of AMPAR trafficking or transmission (Liu *et al*. 2018; Ning *et al*. 2004; Park *et al*. 2008).

Yang *et al*. (2014) wanted to further explore PTEN as a therapeutic target for neurodegenerative disorders. They developed induced pluripotent stem cell (iPS) -derived, motor neuron model, capable of expressing AMPARs. PTEN KD in both in these iPS-derived cells and cultured neurons reduced expression of GluA1 and GluA2, and AMPAR-mediated basal currents, which can lead to in AMPAR-induced cell death (Yang *et al*. 2014). Yang *et al*. suggest PTEN is therefore a potentially useful target for neuronal protection in neurodegeneration in motor neurons, via reducing excitatory transmission inhibition. These results are congruent with research in corticospinal tract (CST) regeneration; PTEN deletion promotes axonal regeneration in retinal nerve cells after injury (Park *et al*. 2008). It was suggested that this regeneration was due to an increase of signalling through the PI3K/ Mammalian Target of Rapamycin (mTOR) pathway (Park *et al*. 2008).

SMA is caused by deletion/mutation of *survival motor neuron* (*SMN*) gene (Lefebvre *et al*. 1995), so SMN-deficient motor neurons can be used as a SMA model (Ning *et al*. 2010). PTEN KD enhanced axonal elongation and growth cone size, which increased cell survival in these cells. These effects were associated with increases in p-Akt, as well as restoration of β-actin levels (Ning *et al*. 2010). Enhanced survival was not seen when cells were also treated with PI3K inhibitor LY294002, suggesting the importance of the PI3K/Akt pathway. When adenovirus expressing PTEN KD siRNA was injected into p1 SMN-mutant mice, these mice showed improved motor neuron survival; these results together also show the importance of PTEN in neurodegneration

and its potential use as a therapeutic target (Ning *et al*. 2010). Taken together, these studies show that modulation of PTEN can be neuroprotective in a range of neurodegenerative disorders, through mechanisms including AMPAR trafficking, cell death and synaptic transmission.

1.5.10 The Autophagy–Lysosomal Pathway and Aβ Metabolism

Deficits in the endosome-lysosome system are strongly implicated in neurodegeneration (Mayer *et al*. 1994; Kimura and Yanagisawa 2018; Vagnozzi and Pratico 2019; Martini-Stoica *et al*. 2016). Retromer is necessary to sort cargo proteins to be trafficked from endosomes to the Golgi and is therefore a vital part of this system (Cullen and Korswagen 2012; Seaman 2012). Due to their finding that AD *post-mortem* brains show up 32 times larger endosomes than controls, Cataldo *et al*. (1997) suggest that in AD there is increased endocytic activity and trafficking to endosomes, which can increase β-amyloidogenesis under certain conditions, due to rerouting of proteases able to cleave Aβ. Due to evidence that APP cleavage can result in Aβ creation through endocytic processes, Kimura and Yanagisawa *et al*. (2018) suggest the “traffic light” hypothesis, which suggests that AD pathology is in part caused by changes in the metabolization of Aβ and tau due to dysregulation of the endocytic pathway (Kimura and Yanagisawa 2018; Cataldo *et al*. 2001; Cataldo *et al*. 1997). Further evidence for this will be outlined below, along with evidence of other routes by which retromer making be involved in neurodegeneration.

1.5.11 Retromer in Neurodegeneration

Due to recent evidence implicating PTEN in retromer-mediated trafficking (Shinde and Madikka, 2017), this thesis will also aim to further examine how PTEN can regulate retromer. Retromer is also reported to be involved in plasticity, and may be relevant in neurodegeneration, via trafficking and metabolism of Aβ, and through effects on AMPARs and neurotransmission (Small *et al*. 2005; Muhammad *et al*. 2008; Temkin *et al*. 2017; Bhalla *et al*. 2012; Vilarino-Guell *et al*. 2011).

VPS35 is found in the soma, axon and dendritic spines in neurons, and retromer is involved in trafficking of many neuronal proteins including β2 adrenergic receptors (β2ARs), AMPARs, NMDARs and dopamine transporters (Munsie *et al*. 2014; Choy *et al*. 2014; Cai *et al*. 2011; (Wu *et al*. 2017). There is direct evidence for the role of retromer in neurodegeneration pathology through separate mechanisms involving AMPAR trafficking and Aβ metabolism (Mecozzi *et al*. 2014; Temkin *et al*. 2017; Bhalla *et al*. 2012; Vilarino-Guell *et al*. 2011).

1.5.12 Retromer in Neurodegeneration- APP Processing

Mecozzi *et al*. (2014) showed evidence for the role of retromer in APP processing. They found a compound called R55, which stabilised retromer, could reroute APP away from the endosome, reducing pathogenic APP cleavage in hippocampal neurons with double APP mutation (Mecozzi *et al*. 2014). $A\beta_{40}$ and $A\beta_{42}$ levels were significantly reduced, which Mecozzi *et al*. (2014) suggest is due to reduced APP processing by BACE1. This was confirmed by measuring β-CTF, a product of BACE1 cleavage of APP (Cai *et al*. 2001).

These were significantly lowered by R55 treatment. Furthermore, R55 increased sAPPα levels in the medium, which Mecozzi *et al*. (2014) explain is consistent with APP processing through the α-secretase pathway when BACE1 processing is reduced (Mecozzi *et al*. 2014, (Luo *et al*. 2001). This study shows that retromer is critical in the fate of APP in the sense that it is highly implicated in which pathway it is processed by (Mecozzi *et al*. 2014).

Furthermore, VPS35 haploinsufficiency can increase APP cleavage in AD mouse model Tg2576, which express a double-mutant form of APP (K670N/M671L), exhibit 14 times more $A\beta_{1\text{-}42/43}$ and show age-related memory deficits (Hsiao *et al*. 1996; Wen *et al*. 2011). Deletion of one VPS35 allele in Tg2576 mice was associated with earlier-onset AD related pathology, including reduced LTP and impairment of spatial learning and memory. AMPAR and NMDAR mini EPSCs were both reduced in Tg2576 mice with VPS35 hemizygous deletion compared to WT or Tg2576 mice, and these mice also showed enhanced $A\beta_{40}$ levels compared to Tg2576 or WT mice (Wen *et al*. 2011). VPS35 associates with BACE1 in mouse brain and during VPS35 depletion, BACE1 showed translocation away from the Golgi and increased colocalization with LAMP1 an endosome/lysosomal marker (Wen *et al*. 2011). It was suggested that VPS35 is therefore necessary for BACE1 localisation in the Golgi and retrieval from endosomes (Wen *et al*. 2011). There were higher levels of BACE1 activity, shown using a BACE1 assay kit and increase in products associated with BACE1 cleavage such as Soluble amyloid precursor protein alpha (sAPPβ) in the Tg2576/VPS35 deletion mice compared to Tg2576 (Wen *et al*. 2011). In conclusion, retromer is strongly implicated in APP processing through regulation of BACE1 localisation, and deficits in this mechanism have influence over neurotransmission and learning and memory in AD mouse models (Wen *et al*. 2011).

Recently, Ansell-Schultz *et al*. (2018) found that Aβ oligomers accumulated when VPS35 was diminished; Aβ oligomers also co-localise with VPS35 in early endosomes. Aβ oligomer application reduces expression of VPS35, both in cells which absorbed Aβ oligomers directly

and in cells which took up Aβ oligomers from neighbouring cells (Ansell-Schultz *et al*. 2018). This study suggests a bi-directional relationship between Aβ and retromer; retromer seems to be involved in Aβ distribution, and Aβ can disrupt retromer components (Ansell-Schultz *et al*. 2018).

Furthermore, KD of VPS35, which is found in spines, endosomes and the TGN, reduces the frequency of APP transport and increases APP levels in early endosomes in processes of neurons (Bhalla *et al*. 2012). VPS35 KD also led to enhanced co-localisation of APP and BACE1, causing swelling of early endosomes, as well as increased Aβ levels (Bhalla *et al*. 2012). After synthesis in the ER, BACE1 is trafficked through the Golgi, then a proportion of it moves to the plasma membrane (Tan and Evin 2012; Tan *et al*. 2020). It then is engulfed by early endosomes and is recycled through the to the Golgi, a process which is controlled by retromer (Tan and Evin 2012; He, Li, *et al*. 2005) Inhibiting VPS26 expression leads to BACE1 aggregation in early endosomes (He, Li, *et al*. 2005).

SNX27 can also influence Aβ through regulation of PS1 activity (Wang *et al*. 2014). SNX27 associates with PS1, reducing its activity. SNX27 suppression therefore leads to increased γ-secretase activity and subsequent Aβ production; while SNX27 over-expression reduces Aβ generation via γ-secretase inhibition in AD mouse model Tg2576 (Wang, Huang, *et al*. 2014). Furthermore, Sorting-related Receptor with A-type Repeats (SORLA) regulates APP processing; SORLA KO in mice leads to enhanced Aβ levels in the brain (Andersen *et al*. 2005). SORLA interacts with the PDZ domain of SNX27, and SNX27 overexpression can increase surface expression of APP and SORLA in neurons, and can also increase sAPPα generation (Huang *et al*. 2016).

1.5.13 Retromer in Neurodegeneration – AMPAR Trafficking

There is strong evidence for the role of retromer in AMPAR trafficking (Munsie *et al*. 2014; Tian *et al*. 2015; Temkin *et al*. 2017). Retromer-mediated AMPAR trafficking may be relevant in neurodegeneration pathology; VPS35 mutations are associated with defective AMPAR trafficking and excitatory neurotransmission in PD models (Munsie *et al*. 2014). D620N is a loss-of-function mutation found in PD that blocks the ability of VPS35 to localise in spines (Munsie *et al*. 2014). D620N overexpression leads to altered surface AMPAR expression in cortical neurons compared to WT VPS35; neurons expressing this mutant show enhanced GluA1 synaptic clustering (Munsie *et al*. 2014). This is also seen in dopaminergic neurons derived from induced pluripotent stem (iPS) cell from fibroblasts from patients with this mutation. This increase clustering is attributed to two potential causes: reduction in GluA1 recycling at the membrane, or/and increased immobile GluA1 (Munsie *et al*. 2014).

Expression of this mutant also enhances excitatory synaptic transmission in cortical neurons (Munsie *et al*. 2014). Tsika *et al*. (2014) report that expression of this mutant, compared to WT, impairs neurite outgrows in cortical neurons and leads to axonal degeneration of dopaminergic neurons.

These studies together provide evidence that retromer is involved in processes relevant to neuronal function and neurodegeneration including neurotransmission, receptor trafficking and Amyloid metabolism (Temkin *et al*. 2017; He *et al*. 2005; Vagnozzi and Praticò, 2019; Bhalla *et al*. 2012; Munsie *et al*. 2014; Vilarino-Guell *et al*. 2011, Ansell-Schultz *et al*. 2018).

2 Aims

The purpose of this work is to examine the role of PTEN in membrane protein trafficking, which is an important aspect of synaptic plasticity. The role of ubiquitination and SUMOylation on PTEN in this context will also be tested.

The main aims are:

- To create and test tools to study PTEN SUMOylation/ ubiquitination
- To characterise these tools and examine how to changes to PTEN SUMOylation regulate its function
- To test cross-regulation of SUMO and ubiquitin on PTEN
- To test the role of PTEN and PTEN SUMOylation/ ubiquitination in AMPAR trafficking
- To examine how PTEN affects retromer in terms of its trafficking ability and regulation of its components, and if SUMOylation/ ubiquitination are involved

Materials and Methods

3 Materials and Methods

3.1 Culture of HEK and HeLa cells

3.1.1 Passaging and Storage

HEK293T and HeLa cells were grown in an incubator at 37 ºC 5% CO_2 and cultured in Dulbecco's Modified Eagle's Medium (DMEM; D5796 Sigma) containing 5% Fetal Bovine Serum (Gibco). When confluent, cells were split into a new flask. Cells were washed twice in PBS, (10x stock from Gibco), trypsinised by addition of 0.1% trypsin-EDTA (Sigma) for five minutes, then fresh media was added and cells were centrifuged for 2 minutes at 1600 RPM. The media was then aspirated, and cells were suspended in fresh media. 10% of the total cells were added to a new T75 flask and kept as stock. For transfections, cells were stained with Trypan blue (Sigma) and counted, then split into dishes. HEK-293T cells were used until they had been passaged 25 times.

3.1.2 Plating Cells

6cm plastic dishes (Cellstar) were treated with 2mL 0.1mg/mL Poly-L-Lysine (PLL) (Gibco) for 2 hours at 37°C and washed three times with sterile water. PLL treatment was used to increase adherence of cells to the dish (Sun *et al*. 2012). HEK239T and HeLa cells were trypsinised and resuspended and dissociated cell mix was counted in a haemocytometer.

3.1.3 Transfection

All immunoprecipitation experiments used one 6cm dish of confluent, transfected HEK cells per condition. 1.5 million HEK293T cells were split into each dish. The next day, cells were transfected with the relevant DNA and Lipofectamine 2000 (Invitrogen) (1.5x amount of µg DNA added). DNA and Lipofectamine mixtures were diluted in 500µl plain DMEM in Eppendorfs, vortexed and left for 30 minutes at RT. They were then added dropwise to cells and dishes were gently swirled, and then incubated. Cells were approximately 80% confluent when transfected. Experiments were carried out two days after transfections. For experiments involving MG132, this was added 6 hours before lysis at a concentration of 20µM.

3.2 Culture of Neurons

3.2.1 Dish Preparation

Six well plates (Cellstar) were left overnight in 0.5 mg/ml Poly-L-Lysine, diluted in borate buffer (10mM sodium tetraborate and 50mM boric acid; Sigma). Plates were then washed three times in cell culture water and plating media added (Neurobasal (Gibco) with 2% B27 (Gibco), 5% horse serum (Lab Tech/ Sigma), 1% Glutamax (ThermoFisher) and 1% Penicillin/Streptomycin (Gibco)).

3.2.2 Dissection and Plating

Pregnant Wistar rats were anesthetised according to Schedule 1 procedures and their E18 embryos removed. Brains were removed from embryos and dissected in a 10cm dish containing Hanks Balanced Saline Solution (HBSS; Gibco) at RT. Hindbrain and meninges were discarded and cortices and hippocampus separated. Pieces of cortex and hippocampus were washed three times in 30ml HBSS in a 50ml falcon tube, and trypsinised in a water bath at 37 ºC (hippocampus for 9 minutes in 9ml HBSS with 1ml trypsin and cortex for 9 minutes in 27ml HBSS with 3ml trypsin). Cortices and hippocampus were then washed again three times with 30ml HBSS, then once in 5ml plating media. Cells were then dissociated by pipetting (using a P100 for hippocampus and 5ml serological pipette for cortex). Dissociated cells were suspended in warm plating media to a total volume of 20 ml for cortical cells and 5ml for hippocampal cells. A sterile 70µm nylon mesh filter (Sigma) was used to filter cortical cell suspension. A sample of the cell suspension was diluted 1:10 in Trypan Blue and counted on a haemocytometer. Hippocampal cells were plated into plating media in 15mm dishes on coverslips for imaging, and cortical cells were plated in 2ml plating media at a density of 600,000 in each well of a 6-well plate for biochemistry. The next day, media was replaced with 3ml feeding media (Neurobasal containing 2% B27, 1% Penicillin/Streptomycin (all Gibco) and 0.4% Glutamax (Thermofisher)). For imaging, hippocampal neurons were plated at 200,000 cells per coverslip in a 35mm dish in 2ml plating media. Cells were incubated at 37 ºC with 5% CO2. The next day, media was changed to feeding media.

3.2.3 Neuronal Transfection

1ml transfection media (Neurobasal with 0.4% Glutamax and 2% B27) was added to wells of a six well dish and incubated at 37°C. A tube of 1µg DNA in 100µl plain Neurobasal and a separate tube of 1.5µl Lipofectamine in 100µl were prepared, vortexed and left at RT for 5 minutes. These were then mixed and vortexed again, then left at RT for 30 minutes. Each

coverslip was washed in a dish containing plain neurobasal, then added to the transfection media dish. The DNA mixture was vortexed again then added to the well of the six well dish. This was incubated for 45 mins at 37°C. After incubation, coverslips were washed in plain media, then returned to their original dishes.

3.2.4 Viral Transduction

Lentivirus was defrosted at RT from -80ºC, then warmed in a 37ºC water bath. The relevant virus was added and cells returned to incubator.

3.3 Western Blot

3.3.1 SDS-Page

(See tables below for complete supplier information) Immunoprecipitation experiment samples were centrifuged for 2 minutes before loading. All other samples were gently mixed before loading. 5µl PageRuler™ Prestained Protein Ladder (Thermo) was added to 1 or more wells of each gel. Samples were then loaded into remaining wells of 10% SDS-PAGE gels consisting of a stacking gel at the top (125 mM Tris-HCl pH 6.8, 4% acrylamide, 0.1%, SDS, 0.1% APS and 0.01% TEMED) and a resolving gel at the bottom (10% acrylamide, 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS, and 0.01% TEMED). A 15-well comb was used to separate wells of the stacking gel. Gels were made in 1.5 mm glass plates and allowed to set for an hour before loading. Gels were inserted into electrode holders in Bio-Rad electrophoresis tanks with SDS-PAGE running buffer (25 mM Tris, 250 mM Glycine and 0.1% SDS) and run at 100-150 volts for around 1-1.5 hours until all the dye front had passed through the bottom of the gel. Proteins on gels were then transferred to membranes in electrode cartridges with blotting paper and sponges either side. Tanks were filled with transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol) and an ice block and stirring bean were added. Transfer was run for 66 minutes at 400mA. Membranes were washed once in Phosphate Buffered Saline and Tween (PBST; 137mM NaCl, 10mM Na_2HPO_4, 2.7mM KCl, 2mM K_2HPO_4, 0.001% Tween) then blocked for one hour in 5% milk or BSA in PBST at RT, then incubated with the relevant primary antibody at 4ºC (see Table 2 for antibodies and time of incubation.) After the required length of time, membranes were washed in PBST. Horseradish peroxidase-tagged secondary antibody was added in 5% milk or BSA in PBST at 1:10,000. Membranes were then washed five times in PBST, then 3 times for 5 minutes on a shaker. Membranes were then gently dried of excess PBST and enhanced chemiluminescence substrate (ECL) was added for 60-90 seconds. Membranes were then sealed in clear plastic to prevent drying. They were imaged in a Li-core Odyssey

machine. All blots were developed in this way except Figure 4.3.2 which was developed on X-ray film in a Konica SRX-101A medical film developer in a dark room.

3.3.2 Lysis of Cells for Western Blotting – Total Protein

Confluent cells in 6-well dishes were lysed in 200µl 1x Laemmli buffer (see below), scraped into tubes and boiled on a heat block at 95°C for 10 minutes. In the case of neurons, cells were lysed at a specific number of days in vitro (DIV) as specified. Samples were stored up to a week at 4°C or for longer periods at -20°C.

3.3.3 Laemmli Sample Buffer (4x)

Buffer containing 4% SDS, 10% glycerol, 0.004% bromophenol blue, 125 mM Tris pH 6.4. For lysis for total protein experiments, this was diluted to 1x. For adding to beads after immunoprecipitation experiments, 2x was added. 10% 2-β-mercaptoethanol (βME) was added fresh each time before use.

Table 2. Antibodies

Antibody	**Cat. No./ Clone**	**Supplier**	**Diluent**	**Conc.**	**Secondary Antibody**	**Time**
β-Actin	A5441	Sigma-Aldrich	Milk	1:10,000	Rabbit	1 hour
Akt	PAN40D4 (2920S)	Cell-Signalling Technologies	BSA	1:1000	Mouse	Overnight
ASCT2	8057 (D7C12)	Cell-Signalling Technologies	Milk	1:1000	Rabbit	Overnight
Phos Akt S473	D9E (4060S)	Cell-Signalling Technologies	BSA	1:1000	Rabbit	Overnight
GAPDH	ab8245	Sigma	Milk/BSA	1:10,000	Mouse	1 hour
GFP	Pabg1-100	ChromoTek	Milk/BSA	1:4000	Rat	1 hour
GluA1	13185 (D4N9V)	Cell Signalling Technologies	Milk	1:1000	Rabbit	Overnight
GluA2	182103	Synaptic Systems	Milk	1:1000	Rabbit	Overnight
GLUT1	ab115730	Abcam	Milk	1:1000	Rabbit	Overnight
HA	HA-7 H3663	Sigma	Milk/BSA	1:1000	Mouse	1 hour
VPS26	ab23892	Abcam	BSA	1:1000	Rabbit	1 hour
VPS35	ab95745	Abcam	BSA	1:1000	Mouse	Overnight
GST	27457701V	GE Healthcare	Milk	1:500	Goat	1 hour
Myc	9B11	Cell-Signalling Technologies	Milk	1:4000	Mouse	1 hour
PTEN	95525	Cell Signalling Technology	Milk/BSA	1:1000	Rabbit	1 hour
SBP	10764	Millipore	Milk	1:4000	Mouse	1 hour
SENP3	5591S	Cell Signalling Technology	BSA	1:1000	Rabbit	1 hour
SUMO1	4930S	Cell-Signalling	BSA	1:1000	Rabbit	Overnight

		Technologies				
SUMO2/3	4971S	Cell-Signalling Technologies	BSA	1:1000	Rabbit	Overnight
Ubiquitin (P4D1)	3936	Cell-Signalling Technologies	Milk	1:1000	Mouse	1 hour
SNX27 (detection in cell lines)	ab77799	Abcam	Milk	1:2000	Mouse	1 hour
SNX27 (detection in rat neurons)	N/A	A gift from Dr Martin Playford, National Institute of Health, USA	Milk	1:1000	Rabbit	Overnight
Transferin Receptor	sc-65882 H68.4 (CD71)	Santa-Cruz	Milk	1:1000	Mouse	Overnight
N-Cadherin	13A9	Cell-Signalling Technologies	BSA	1:1000	Mouse	Overnight
Phospho-ERK (ERK1/ 2)	E7028(ERK1 T202/T204 and ERK2 T185/T187)	Sigma	BSA	1:1000	Rabbit	Overnight

Horseradish peroxidase (HRP)-conjugated secondary antibodies for Western blotting (anti-rat raised in rabbit, anti-mouse and anti-rabbit raised in goat) were sourced from Sigma and used at 1:10,000 concentration in the same diluent in which the primary had been used (5% milk or BSA in PBST). Cy3 secondary antibody for confocal imaging (chicken, raised in donkey) was sourced from Jackson ImmunoResearch and used at 1:400 in 2% BSA in PBS.

Table 3. Reagents- SDS-PAGE and Western Blot

Reagent	Description	Supplier
Milk powder	For blocking and blotting membranes	The Co-Operative
Pageruler™	Prestained Protein Ladder for SDS-PAGE	Thermo-Fisher
30% Acrylamide	For SDS-PAGE gels	Geneflow Limited
TEMED	Tetramethyl-ethylenediamine	Sigma-Aldrich
APS	Ammonium Persulfate	Sigma-Aldrich
BSA	Bovine Serum Albumin	Sigma-Aldrich
0.45 µm Immobilon-PVDF	Polyvinyl difluoride membrane	Millipore
NEM	N-Ethylmaleimide	Sigma-Aldrich
BME	2-Mercaptoethanol	Sigma-Aldrich
Triton-X 100-X	Detergent	Sigma-Aldrich
SDS	Sodium dodecyl sulphate	Sigma-Aldrich
Tris-HCL	TRIZMA base T1503 + dh20 + HCL	Sigma-Aldrich
SuperSignal™	Enhanced chemiluminescent substrate: Pico and Femto	Thermo-Fisher
Immobilon	Enhanced chemiluminescent substrate: Classico and Forte	Millipore

3.4 Immunoprecipitation Experiments

3.4.1 PSD-95 and SNX27 Immunoprecipitation – GFP Trap

See tables below for supplier information- cells were lysed in 0.7 ml ice cold buffer containing 50mM Tris-HCL pH 7.4, 150mM NaCl, 0.5% Triton-X 100, 20µM NEM (Sigma), and protease inhibitors (Roche). Samples were vortexed, left on ice for 25 minutes, then centrifuged in a benchtop centrifuge for 20 minutes at 4ºC at 13.2 rpm. The supernatant was removed and a 20µl input sample taken from each sample and kept on ice. The remaining supernatant was added to 6µl GFP-Trap beads (Chromotek) and samples were left at 4ºC on a rotating wheel at for one hour. Samples were then washed three times in 1ml wash buffer containing 50mM Tris-HCL pH7.4, 150 mM NaCl and 0.5% Triton-X 100. Samples were centrifuged at 4000rpm for 2 minutes at 4ºC between each wash to pellet beads. 2X Laemmli buffer was then added to inputs and samples and all were heated on a shaking heat block at 95 ºC for 10 minutes.

3.4.2 SUMO and Ubiquitin Immunoprecipitation – GFP Trap

Two days after transfection, cells were lysed in 0.7ml ice cold buffer containing 20mM Tris-HCL pH 7.4, 137mM NaCl, 2mM EDTA, 1%Triton-X 100-X, 10% Glycerol, 0.1% SDS, 20µM NEM and protease inhibitors and scraped. Samples were sonicated to enhance likelihood of SUMOylation detection, as sonication can improve immunoprecipitation of proteins which can be hindered by viscous components in lysate (Meng et al, 2017). Each Eppendorf was subject to 5x 5 second pulses using a Microson Ultrasonic Cell Disrupter on the highest setting (5), they were then left on ice for 25 minutes and centrifuged in a benchtop centrifuge for 20 minutes at 4ºC at 13.2 rpm. The supernatant was removed and a 20µl input sample taken from each sample and kept on ice. The remaining supernatant was added to 6µl GFP-Trap beads and samples were left at 4ºC on a rotating wheel at for one hour. Beads were then washed three times in the same buffer as the lysis buffer, without protease inhibitors and NEM. 2x Laemmli buffer was added and samples were boiled at 95ºC for 10minutes. For the ubiquitin immunoprecipitation, 10µM MG132 was added for 6 hours prior to lysis.

3.4.3 SUMO2/3 Immunoprecipitation in 2% SDS – GFP Trap

The same protocol as above was followed, with same lysis buffer but with 2% SDS during lysis. Before being added to beads, lysate was diluted with lysis buffer without SDS so that final concentration of lysate on beads contained 0.5% SDS.

3.4.4 SUMO2/3 and Ubiquitin Immunoprecipitation with SENP1 – GFP Trap

Cells were lysed in 0.7ml ice cold buffer containing 20mM Tris-HCL pH 7.4, 137mM NaCl, 2mM EDTA, 1%Triton-X 100-X, 10% Glycerol, 0.5% SDS, 20µM NEM and protease inhibitors. Samples were sonicated, left on ice for 25 minutes, then centrifuged in a benchtop centrifuge for 20 minutes at 4ºC at 13.2 RPM. The supernatant was removed and a 20µl input sample taken from each sample and kept on ice. The remaining supernatant was added to 6µl GFP-Trap beads and samples were left at 4ºC on a rotating wheel at for one hour. Samples were washed three times in wash buffer containing 50mM Tris-HCI pH 7.4, 150mM NaCl and 5mM $MgCL_2$, pH 7.5. Recombinant SENP1 enzyme was added at a final concentration of 100nM diluted in wash buffer, the same amount of buffer was left on the other samples not treated with SENP1. All samples were incubated at 37ºC for an hour. 2x Laemmli buffer was then added to all samples and inputs and they were boiled at 90ºC for 10 minutes.

3.4.5 PTEN Dimer Immunoprecipitation

Cells were lysed in 0.7mL ice cold buffer containing 50mM Tris-HCL pH 7.4, 150 mM NaCl, 0.5% Triton-X 100, 20µM NEM, and protease inhibitors. Samples were vortexed, left on ice for 25 minutes, then centrifuged in a benchtop centrifuge for 20 minutes at 4ºC at 13.2 rpm. The supernatant was removed and a 20µl input sample taken from each sample and kept on ice. The remaining supernatant was added to 6µl GFP-Trap beads and samples were left at 4ºC on a rotating wheel at for one hour. Samples were then washed three times in 1ml wash buffer containing 50mM Tris-HCI pH7.4, 150 mM NaCl and 0.5% Triton-X 100. Samples were centrifuged at 4000rpm for 2 minutes at 4ºC between each wash to pellet beads. 2X Laemmli buffer containing 10% BME was then added to inputs and samples and all were heated on a shaking heat block at 95 ºC for 10 minutes.

3.4.6 Cycloheximide Timepoint Experiments

The day after splitting, wells of a 12 well dish of HEK293T cells were transfected with 1µg GFP-tagged WT-PTEN or 3KR-PTEN DNA. The next day, cycloheximide was added to media at a concentration of 100µg/ml. 24 hours later, cells were lysed in 1x Laemmli buffer.

3.4.7 Surface Biotinylation - Neurons

Wells of 6 well plates of neurons were biotinylated, with 600,000 cells in each well for each condition. Plates were cooled on ice for 2 minutes, then kept on ice for the rest of the protocol. After cooling, cells were washed three times in PBS, then 0.3mg/ml Sulpho-NHS-SS-biotin (Thermo-Fisher) in PBS was added for 10 minutes. Cells were then washed five times in PBS and lysed in buffer containing 50mM Tris pH 7.4, 150mM NaCI, 1% Triton-X 100, 0.1% SDS, and protease inhibitors. All steps were carried out on ice and all buffers were kept on ice. After cells were lysed, samples were vortexed and left on ice for 25 minutes, then centrifuged for 20 minutes at 4ºC at 13.2 RPM. A 20µl input sample taken from each sample and kept on ice. Equal amounts of the remaining supernatant were then added to 30µl Streptavidin beads (Sigma) and tubes were left at 4ºC on a rotating wheel at for one hour. Samples were then washed three times in 1ml wash buffer (lysis buffer without protease inhibitors). Samples were centrifuged at 4000rpm for 2 minutes at 4ºC between each wash to pellet beads. 2X Laemmli buffer containing 10% BME was then added to inputs and samples and all were heated on a shaking heat block at 95 ºC for 10 minutes.

3.4.8 Surface Biotinylation - HeLa cells

250,000 stable HeLa cells expressing Scr, PTEN KD, PTEN-WT, PTEN-3KR and PTEN CM were plated into each well of a 6-well plate. The next day, cells were washed three times in PBS, then 0.3mg/ml Sulpho-NHS-SS-biotin in PBS was added for 10 minutes. Cells were then washed five times in PBS and lysed in buffer containing 50mM Tris pH 7.4, 150mM NaCl, 1% Triton-X 100, 0.1% SDS and protease inhibitors. All biotinylation steps were carried out on ice and all buffers were kept on ice. After cells were lysed, samples were vortexed and left on ice for 25 minutes, then centrifuged for 20 minutes at 4ºC at 13.2 rpm. A 20µl input sample taken from each sample and kept on ice. The remaining supernatant was added to 30µl Streptavidin beads and samples were left at 4ºC on a rotating wheel at for one hour.

Samples were then washed three times in 1ml wash buffer (lysis buffer without protease inhibitors). Samples were centrifuged at 4000rpm for 2 minutes at 4ºC between each wash to pellet beads. 2X Laemmli buffer was then added to inputs and samples and all were heated on a shaking heat block at 95 ºC for 10 minutes.

Table 4. Biochemistry Reagents

Reagent	Description	Supplier	Cat No.
EZ-Link™ Sulfo-NHS-SS-Biotin	Biotin for surface Biotinylation	Thermo-Fisher	21331
ChromoTek GFP-Trap®	GFP beads	ChromoTek	N/A
SENP Enzyme	SENP1 catalytic domain	Made in the lab by Dr A. Nishimune	N/A
MG132-R	Degradation blocker	Tocris	6033
Complete™ Protease Inhibitor tablets	Protease inhibitors	Roche Diagnostics	11697498001
Streptavidin beads	From *Streptomyces avidinii*	Sigma-Aldrich	GE90100484
NEM	N-Ethylmaleimide	Sigma-Aldrich	E3876
PBS	Phosphate Buffered Saline	Gibco	

3.5 Confocal Imaging

3.5.1 Coverslip Preparation

Coverslips were treated in nitric acid overnight, then washed 3 times in H20 and left on a rotating plate for 30 minutes during each wash. They were then left in ethanol for 4 hours, then washed three times in cell culture water on a rotating plate. Coverslips were then left overnight in 1mg/ml Poly-L-Lysine, diluted in borate buffer (10mM sodium tetraborate and 50mM boric acid, both Sigma). Coverslips were then washed three times in cell culture water and plating media added.

3.5.2 Coverslip Fixing, Antibody Staining and Confocal Imaging

25mm coverslips were placed in a six well dish with 1ml 4% paraformaldehyde (PFA) (Sigma) in 1x PBS facing upwards and incubated for 15 minutes at 37°C. Coverslips were then washed three times in 1x PBS at RT. PFA was then quenched with 100mM glycine in 1x PBS and coverslips washed three times in PBS. 1.5ml TBP (0.1% Triton-X 100, 2% BSA in 1x PBS) was added for 20 minutes at RT. 90µl primary antibody (GFP) was then added in 3% BSA in PBS for 45 minutes, while coverslips face down on parafilm at RT. Coverslips were put back into six well dishes and washed three times in PBS, then put

face down again onto parafilm with 90µl secondary antibody in 3% BSA for 45 mins in the dark. Coverslips were then put back into six well dish, washed four times in PBS and once in ddh20 and fixed onto glass slides with Fluoromount-G mounting medium containing DAPI (4′,6-diamidino-2-phenylindole) nuclear fluorescent stain (eBioscience). Coverslips were imaged with a 63x Harmonic Compound X PL APO CS oil immersion objective on a Leica SP5-II confocal laser scanning microscope. 1024x1024 pixel resolution was used. Zoom parameters were not kept constant due to the preliminary nature of this experiment.

3.5.3 Analysis and Statistics

For, immunoprecipitation experiment samples, 2µl of each sample was run and blotted for the tag of the immunoprecipitated protein. The signal from the immunoprecipitated protein was normalised to this, to account for differences in expression of the recombinant protein between conditions. The blot of the 2µl immunoprecipitated sample is labelled re-probe. ImageStudio software was used to analyse bands for LI-CORE images, ImageJ was used to analyse bands developed on film. Statistical analysis and presentation were carried out in Prism. All error bars represent SEM unless otherwise stated.

3.6 Cloning of DNA Constructs

Table 5. Reagents- Cloning

Reagent	**Description**	**Supplier**	**Cat No.**
Hyperladder (1kb) DNA marker	DNA gel marker	Bioline	BIO-33053
GeneJET Plasmid Miniprep Kit	Miniprep kit	Thermo-Fisher	K0503
GeneJET Plasmid Midiprep Kit	Midiprep kit	Thermo-Fisher	K0481
DNA gel Loading dye (6x)	DNA gel dye	New England Biolabs	B7025
XL1-Blue *E-coli*	Competent cells	Agilent	200236
DMSO	Dimethyl Sulfoxide	Sigma-Aldrich	D8418
KOD Hot Start DNA Polymerase kit (MgSO4, dNTPS, 10x hot start buffer, polymerase)	PCR Reagents	Merk-Millipore	71086
Restriction enzymes		New England Biolabs	
T4 DNA ligase solution1		Takara	2011B
Custom DNA Oligonucleotide primers		Sigma	N/A
Luria broth	Bacterial medium	Fisher Scientific	BP14262
Ampicillin	Ampicillin sodium salt 100mg/ml stock in 50% ethanol	Sigma-Aldrich	A-9518
Kanamycin	Kanamycin monosulfate 10mg/ml stock in Dh20	Sigma-Aldrich	K-4000
Agarose DNA Gel	A-6013 Agarose Type I, Low EEO	Sigma-Aldrich	A6013
GeneJET™ Gel Extraction Kit	DNA extraction	Thermo-Fisher	K0691

Table 6. Plasmids

Construct	***Tag***	***Creator/ Source***	***Vector***	***Cloning Sites and Promotor***	***Notes***
Empty Vectors					
pSUPER-neo-GFP	GFP		-	-	Bacterial expression vector
pXLG3-PX-GFP-WPRE	GFP	Dr K Wilkinson	-	-	Lentiviral vector
ShRNA Constructs					
PTEN shRNA	GFP	Rachel Milligan	pXLG3-PX-GFP-WPRE	H1-shRNA fragment cloned into the PacI and XhoI sites	shRNA target sequence: CGACTTAGACTT GACCTATAT
Scr	GFP	Dr K Wilkinson	pXLG3-PX-GFP-WPRE	H1-shRNA fragment cloned into the PacI and XhoI sites	Control shRNA-expressing plasmid used as a control for the PTEN shRNA. Contains a mock target sequence:AATTCT CCGAACGTGTCA C
S3-Scr	GFP	Dr K Wilkinson	pXLG3-GFP-100bp stuffer	U6-shRNA fragment cloned into the KpnI site	Control shRNA for the SENP3 shRNA. Contains a mock target sequence:GCACTA CCAGAGCTAACT CAGATAGTACT
SENP3 shRNA	GFP	Dr K Wilkinson	pXLG3-GFP-100bp stuffer	U6-shRNA fragment cloned into the KpnI site	shRNA target sequence:TATGGA CAGAACTGGCTC AATGACCAGGT
PTEN O/E Constructs					
TAP-PTEN-WT (Rat)	SBP. CBP (N-term)	Dr Michaela Heimann	pNTAP-B	PTEN cloned into BamHI/XhoI sites. Driven by a CMV promoter	Source of the rat PTEN cDNA sequence used throughout. NCBI entry AF455569.1
TAP-PTEN-3KR (Rat)	SBP. CBP (N-term)	Dr Michaela Heimann	pNTAP-B	PTEN cloned into BamHI/XhoI sites. Driven by a CMV promoter	

SUMO/Ubiquitin Constructs					
HA- SUMO1 (Human)	HA (N-term)	Dr Ruth Carmichael	pXLG3-PX-GFP-WPRE	SpeI/BamHI under the control of an SFFV promoter	
HA- SUMO2 (Human)	HA (N-term)	Dr Ruth Carmichael	pXLG3-PX-GFP-WPRE	SpeI/BamHI under the control of SFFV promoter	
HA-Ubiquitin	HA (N-term)	Lab stocks			
FLAG-Ubc9 (Mouse)	FLAG (N-term)	Dr K Wilkinson	pCMV-FLAG	Cloned EcoRI/XhoI under the control of a CMV promoter	
PDZ Domain Constructs					
MYC-PSD-95	Myc (N-term)	Prof. Seth Grant			
GFP-SNX27 (Human)	GFP (N-term)	Prof. Pete Cullen	pEGFP-C	CMV	
Lentiviral Helper Plasmids					
pMD2.G		Prof. Pete Cullen			Addgene plasmid 12259
p8.91		Prof. Pete Cullen			Addgene plasmid 12263

3.6.1 General Cloning Methods

All plasmids were sequenced by Eurofins Genomics. All PCR reactions were carried out in a MJ Research PTC-200 PCR Gradient DNA Engine Thermal Cycler.

Recipes

KOD Polymerase Reaction

Reagent	*Amount*
10x Buffer*	5µl
dNTPs* (0.2mM each final concentration)	5µl
MgSO4* (1.5mM final concentration)	3µl
Polymerase* (0.02U/µl final concentration)	1µl
10uM forward primer (0.3 µM final concentration)	1.5µl
10uM forward primer (0.3 µM final concentration)	1.5µl
Template DNA (1ng/µl; 10ng total template DNA in reaction)	10µl
dH20	23µl

(* from KOD Hot Start DNA Polymerase Kit; Millipore Novagen)

PCR Cycling

Step	*Temperature*	*Duration*
1. Polymerase Activation	95ºC	2 minutes
2. Denature	95ºC	20 seconds
3. Annealing	55ºC	10 seconds
4. Extension	70ºC	10 seconds for shRNA fragment
5. Repeat steps 2-4	24 cycles	

Designed according to Novagen User Protocol TB34

Digest of Vectors and PCR products

5µl vector DNA was mixed with 0.25µl enzymes, 2µl 10x Cutsmart buffer, 2µl CIP (all NEB) and 12.5µl H20. For PCR products, 45µl was mixed with 10µl Cutsmart buffer, 2µl enzymes and H20 up to 100µl. Digest mixtures were incubated for 1.5 hours at 37°C.

Ligation

Digested PCR products and vectors were run on an agarose gel and imaged on a UV transilluminator to determine approximate relative concentrations. PCR products and vectors were then ligated at an insert: vector ratio of 5:1. PCR products and vector were first diluted in water. 1µl vector was mixed with 1µl PCR product and 2µl Takara 1 solution (Takara). The tube was flicked and incubated for 30 minutes.

3.6.2 PTEN KD Plasmid

PTEN KD shRNA primers were designed with the help of Dr Kevin Wilkinson. These oligonucleotide primers diluted in Tris EDTA buffer, left to mix for an hour then were heated at 95ºC for 4 minutes to anneal them:

GATCCCCCGACTTAGACTTGACCTATATTTCAAGAGAATATAGGTCAAGTCTAAGTCGTT TTTC-3' (see target sequences in table above) and 5'-TCGAGAAAAACGACTTAGACTTGACCTATATTCTCTTGAAATATAGGTCAAGTCTAAGTC

GGGG-3'. The double stranded product was then cloned next to a H1 promotor in the plasmid pSUPER-neo-GFP, between BamHI and XhoI sites. To create a PTEN KD construct in a viral vector, the H1-shRNA section was amplified by PCR using generic primers H1 PacI F (5'-CACTTAATTAACGAACGCTGACGTCATCAACC-3') and M13_30nt R (5'-AGCGGATAACAATTTCACACAGGA-3'). The product was then cloned into lentiviral vector pXLG3-PX-GFP-WPRE between PacI and XhoI sites, creating pXLG3-shPTEN-GFP-WPRE. As a control, a pXLG3-PX-GFP-WPRE vector expressing a non-targeting, scrambled shRNA (mock target sequence 5'- AATTCTCCGAACGTGTCAC-3') under the control of a H1 promoter was used.

3.6.3 GFP PTEN-WT and GFP PTEN-3KR

Dr Michaela Heimann and Dr Kevin Wilkinson originally created this mutant in the plasmid pNTAP-B (Agilent Technologies). The PTEN-3KR mutant has mutations at lysine residues shown to be SUMOylated on PTEN: K266, K254 and K289 (Huang *et al.* 2012, Gonzalez-Santamaria *et al.* 2012, Bassi *et al.* 2013). These were mutated to arginine.

Dr Kevin Wilkinson created a GFP-tagged PTEN-3KR mutant via the following methods. He also made a GFP-tagged PTEN-WT clone using the PTEN-WT rat sequence (NCBI entry: AF455569.1). The following methods were conducted by Dr K Wilkinson: First, shRNA-resistant GFP-PTEN-WT and PTEN-3KR rescue constructs were made by using site-directed mutagenesis to make 6 silent mutations in the shRNA sequence of pNTAP-B. This was done with primers: shPTEN-res F (5'-CAAGAGGATGGATTCGACCTGGATCTAACATATATTTATCCAAATATT-3') and shPTEN-res R (5'- AATATTTGGATAAATATATGTTAGATCCAGGTCGAATCCATCCTCTTG-3').

These constructs were then amplified by PCR using the primers PTEN F (5'-GTGGGATCCACAGCCATCATCAAAGAGATC-3') and PTEN R (5'-CACTGGCCATCAGACTTTTGTAATTTGTGA-3'), creating GFP-PTEN-WT and PTEN-3KR rescue constructs. A digestion was then carried out on the PCR product and pXLG3-shPTEN-GFP-WPRE vector using BamHI and MscI and the PCR product was cloned into the vector.

3.6.4 Bacterial Transformation and DNA Preparation

To amplify DNA plasmids for midiprep, 1µl of DNA was mixed with 30µl of XL-1 Blue *E. coli* and kept on ice for 20 min before 45 second heat shocked at 37ºC. The mixture was kept on ice for 2 minutes and added to 100ml Luria Bertani (LB) broth for 24 hours at 37ºC with antibiotics (Ampicillin or Kanamycin). Kanamycin resistant plasmids were first mixed with 100µl plain broth then incubated at 37 ºC for an hour to allow antibiotic resistance before

being added to broth with Kanamycin. 24 hours later, cultures were midiprepped using GeneJET™ Midiprep Kit to isolate the relevant DNA according to manufacturer's protocol.

3.6.5 Bacterial Strains

For cloning and DNA amplification, Competent DH5α were used. These were made in house.

DH5α (supE44 Δlac Φ80 lacZΔ M15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 u169)

For cloning and DNA amplification of Lentiviral plasmids, XL1-Blue Competent Cells were used. (XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacIqZΔM15 Tn10 (Tetr)])

3.6.6 Agar Plates and Broth for Bacterial Growth

Agar plates were made using 1.5% agar and LB broth and stored at 4°C. Bacteria were grown in LB broth. Both were used with antibiotics kanamycin (25µg/ml) or ampicillin (100µg/ml) and made in house by University of Bristol Technician Team.

3.6.7 Lentivirus Production

To make the SENP3, Scr, PTEN KD, PTEN-WT, PTEN-3KR and PTEN CM Lentivirus, 7 million HEK293T cells were split into 10cm dishes with DMEM and 5% FBS (1 dish per virus). The next day, media was removed and cells were transfected. The following DNA was added to 2.5ml plain DMEM:

- 10µg XLG viral vector
- 2.5µg pMD2.G (expresses VSV-G envelope)
- 7.5µg p8.91 (helper vector)

2.5ml plain DMEM was mixed with 120 µl 1mg/ml PEI (Sigma) and this was added to the DMEM/ DNA mixture. Transfections were left for 30 minutes at RT then added to the cells after media had been removed. Four hours later, transfection mix was replaced with complete DMEM. 36-48 hours later, virus-containing media was centrifuged to remove dead cells, virus was then aliquoted and stored at -80ºC.

3.6.8 HeLa Cell Line Production

40,000 HeLa cells were split into a 12 well dish. The next day, 250µl of each of Scr, PTEN-KD, PTEN-WT, PTEN-3KR or PTEN-C124S Lentivirus were added. Two days later, cells were split into T75 flasks and allowed to grow until confluent, and kept for four passages.

Results Chapter 1

Detecting PTEN SUMOylation and Ubiquitination

4 Detecting PTEN SUMOylation and Ubiquitination

4.1 Introduction

4.1.1 PTEN SUMOylation Sites and Recombinant SUMO in Previous Research

PTEN is reported to be SUMOylated, which has effects on its localisation, phosphatase activity and stability (Huang *et al*. 2012; Bawa-Khalfe *et al*. 2016), but PTEN SUMOylation in the context of neurons or neurodegeneration has not yet been tested. This is of interest due to evidence PTEN attenuation can relieve AD-related synaptic deficits (Knafo *et al*. 2016). Furthermore, other PTMs such as ubiquitination can influence PTEN stability, which is thought to underlie PTEN loss seen in AD (Kwak *et al*. 2010). (For a more detailed analysis of the current literature on PTEN SUMOylation, see General Introduction, section 1.3.).

PTEN has been shown to be SUMOylated at three sites: K254, K266 and K289 (Huang *et al*. 2012; Gonzalez-Santamaria *et al*. 2012), and SIM sites on PTEN have also been identified (Bawa-Khalfe *et al*. 2016). It was reported that PTEN-SUMOylation enables membrane localisation and nuclear retention of PTEN, which has effects on its ability to regulate p-Akt and limit tumorigenesis (Huang *et al*. 2012). It should be noted that most studies that previously tested the level of SUMOylation of PTEN mutants lacking one or more SUMOylatable lysine did so with recombinant, tagged SUMO1 or 2 in immunoprecipitations, or recombinant SUMO in *in vitro* SUMOylation assays (where SUMO proteins are produced in bacteria then purified and mixed with PTEN in a tube, along with the essential enzymes of the SUMOylation cascade), rather than with endogenous SUMO. Overexpression of recombinant SUMO has been suggested to lead to artefacts if it is not controlled (Eifler and Vertegaal, 2015). Furthermore, tagged proteins can behave differently to their endogenous counterparts (Skube *et al*. 2010). Therefore, although the biochemical evidence for the expected reduction in SUMOylation of these non or reduced-SUMOylation mutants in cell lines is compelling, it has so far perhaps not been widely validated in the most physiologically relevant way. Testing endogenous SUMOylation of PTEN is therefore critical in understanding the physiological relevance of SUMOylation.

4.1.2 Evidence for PTEN SUMOylation by Endogenous SUMO

Most of the previous studies have not evaluated the level of endogenous SUMO on PTEN mutants, so from these studies, it cannot be unequivocally concluded how these mutants are affected by endogenous SUMO. The only study to test endogenous SUMO in this way to date was carried out by Bawa-Khalfe *et al*. (2016), who tested SUMOylation in the context of prostate cancer models. A PTEN K254A/K266A double mutant was reported to be less modified by endogenous SUMO1 and ubiquitin than PTEN-WT, in IPs from transfected PC-3

cells (Bawa-Khalfe *et al*. 2016). The group failed to detect endogenous SUMOylation of PTEN with SUMO2/3.

4.1.3 SUMO and Ubiquitin Crosstalk on PTEN

PTEN has also been shown to be ubiquitinated at K13, K66, K266 and K289 (Trotman *et al.* 2007; Gonzales-Santamaria *et al.* 2012, Wu *et al.* 2016). PTEN ubiquitination is important in various aspects of PTEN regulation including stability, catalytic activity and nuclear localisation; some of these effects may be relevant in cancer (Bassi *et al*. 2013; Trotman *et al*. 2007, Leslie and Gupta, 2016; Maccario *et al*. 2010, Duerr *et al*. 1998) and neurodegeneration (Kwak *et al*. 2010). Ubiquitination of PTEN is thought to be involved in the reduced levels of PTEN reported in AD brains (Kwak *et al*. 2010; Griffin *et al*. 2005).

Generally speaking, when target proteins are SUMOylated, the conjugated SUMO can recruit a SUMO targeted ubiquitin ligase (STUbL), which is a ubiquitin E3 ligase that can facilitate ubiquitination (Ohkuni *et al*. 2018). This mechanism allows for cross-talk between SUMO and ubiquitin on target proteins (Geoffroy and Hay 2009; Ohkuni *et al*. 2018). An example is reported by Bawa-Khalfe *et al*. (2016) who showed that in cells lacking SENP1, PTEN associates with WWP2, a ubiquitin E3 ligase. This increased ubiquitination and subsequent degradation of PTEN, so it was concluded that SENP1, through de-SUMOylating PTEN, inhibits SUMO1-Dependent PTEN ubiquitination and degradation (Bawa-Khalfe *et al*. 2016). This mechanism plays a role in prostate cancer development (Bawa-Khalfe *et al*. 2016).

However, SUMO-ubiquitin cross-regulation on PTEN is complex, as SUMOylation has also been found to limit ubiquitination and subsequent degradation of PTEN (Wang *et al*. 2014). It is not clear exactly why this is seen; one explanation suggested by Gonzales-Santamaria *et al*. (2012), is that SUMO and ubiquitin compete for the same sites on PTEN. This is possible given that K266 and K289 are known to be modified by both proteins (Huang *et al*. 2016, Gonzales-Santamaria *et al*. 2012; Trotman *et al*. 2007). Another possibility is that SUMOylation at one site blocks ubiquitination at another site; this is supported by evidence that simultaneous SUMOylation at both sites is not possible, shown by a lack of band shift between single and double mutations at known PTEN SUMOylation sites (Wang *et al*. 2014). Huang *et al*. (2012) explain that more space is needed on PTEN for SUMO modification compared to other PTMs such as phosphorylation, making it difficult for concomitant SUMOylation of K254 and K266 to occur. As ubiquitin is a similar size, it is in theory possible that it is also blocked by SUMOylation at another site.

4.1.4 SUMO-Ubiquitin Hybrid Chains

SUMO and ubiquitin can also form mixed chains on target proteins (Tatham *et al*. 2008). Receptor-associated protein 80 (RAP80), a subunit of a complex containing breast cancer susceptibility gene 1 (BRCA1), mediates recruitment of BRCA1 to sites of DNA damage (Kim, Chen, and Yu 2007; Guzzo *et al*. 2012). The ubiquitin E3 ligase RNF4 can facilitate SUMO-ubiquitin hybrid chains at DNA damage sites, which are necessary for BRCA1 recruitment to these sites (Guzzo *et al*. 2012). SUMO-ubiquitin hybrid chains at DNA damage sites are recognised by RAP80 due the close proximity of a SIM and UIM (Ubiquitin Interacting Motif) on RAP80 (Guzzo *et al*. 2012). The presence of these mixed chains at DNA damage sites increased recognition by RAP80 80-fold, compared to chains formed of SUMO or ubiquitin alone, suggesting the importance of combined SUMO/ubiquitin signals (Guzzo *et al*. 2012). Hybrid chains can also act as a signal for degradation (Tatham *et al*. 2008).

A point of interest in this field is that two sites on PTEN are known to be both SUMOylated and ubiquitinated (K266 and K289) (Trotman *et al*. 2007; Huang *et al*. 2012; Gonzales-Santamaria *et al*. 2012), so it is hard to distinguish which effects are caused by either SUMO or ubiquitin. The Bawa-Khalfe (2016) paper is perhaps most useful for this reason, as they used SENP knockdown and overexpression, and SENPs are specific to SUMO deconjugation (Drag and Salvesen 2008).

4.2 Aims

The goal of the work in this chapter was to create tools to examine SUMOylation and ubiquitination of PTEN, with a view to later examining how SUMO and ubiquitin modification of PTEN may affect plasticity and protein trafficking in neurons and cells lines. It was important to optimise SUMOylation and ubiquitination detection with endogenous SUMO, as this has not been widely tested, and previous studies on SUMO-regulation of PTEN lack homogeneity. I also wanted to characterise SUMO/ubiquitin crosstalk. Specifically, my aims were to

- Optimise the detection of SUMOylation and Ubiquitination of PTEN
- Create and characterise PTEN-SUMO/Ubiquitin mutants as tools to study the effects of PTEN SUMOylation and ubiquitination
- Characterise SUMO/ubiquitin crosstalk on PTEN and examine hybrid chains

4.3 Results

4.3.1 Creation of PTEN KD Lentivirus

To make a PTEN knockdown (KD) Lentivirus, I first cloned a PTEN KD short hairpin RNA (shRNA) into pSuper (an shRNA expression vector). shRNA along with its promoter were then cloned into a GFP expressing viral plasmid (pXLG) (see Methods for all cloning protocols). This was then transfected in HEK cells along with Lentiviral helper plasmids to create Lentivirus (see Methods for virus making protocols). A control virus was also made using a pXLG plasmid containing a scrambled shRNA sequence (Scr), cloned by Dr K Wilkinson. To validate the PTEN KD virus and PTEN antibody, different amounts of PTEN KD or control Scr Lentivirus were added to rat cortical neurons on DIV 7 and left for 7 days. Cells were lysed, subjected to Western blot for PTEN (Figure 4.3.1.1). The blot suggests that PTEN is greatly reduced by the PTEN KD virus in a dose Dependent fashion **(this was later repeated and analysed statistically, see Figure 5.3.7.1),** suggesting the detected band is indeed PTEN and the lentivirus is successfully knocking it down. This Lentivirus is therefore a useful tool to study PTEN.

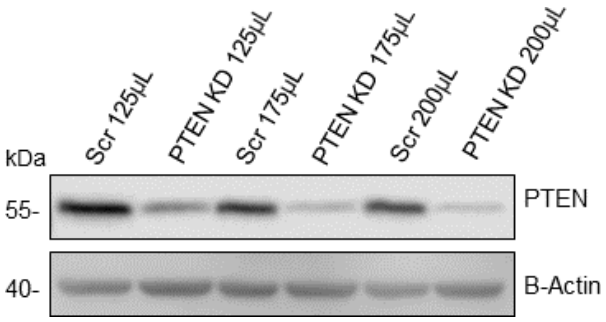

Figure 4.3.1.1. PTEN KD Lentivirus Knocks Down Total PTEN.
Western Blot showing PTEN expression after Lentiviral transduction in neurons. PTEN KD shRNA or Scr viruses were added to wells of a 6 well dish containing rat cortical neurons and 1.5mL media. 7 days later, cells were lysed and subject to Western Blot, then blotted for PTEN and B-Actin (N=1).

4.3.2 Detection of Modified PTEN

In cultured cortical neurons, blotting for PTEN at high exposure reveals a band at around 70kDa, above the usual band detected at 55kDa (Figure 4.3.2.1). A similar band has been detected by Bassi *et al*. (2013), who suggest it is SUMOylated PTEN as it is increased by N-ethylmaleimide (NEM), an inhibitor of cysteine protease enzymes including SENPs, and is decreased by recombinant SENP treatment. The figure shows PTEN signal after two different exposure times; the 55kDa PTEN band is visible, and the higher exposure also shows an additional higher band of PTEN at ~65-70kDa. The absence of this upper band in the PTEN KD Lentiviral condition suggests it is specific to PTEN. However, through further examination of the literature I found that this higher band could also be "PTEN-L", a longer isoform of PTEN (Hopkins *et al*. 2013). Therefore, I stopped using this method as I could not conclude it detected PTEN SUMOylation.

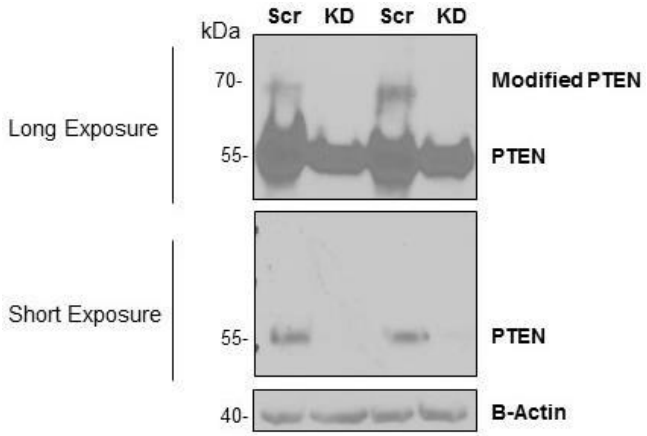

Figure 4.3.2.1 PTEN KD Reduces Modified PTEN.
Cortical rat neurons were transduced with either PTEN KD or Scr Lentivirus. 7 days later, cells were lysed in buffer containing NEM and subject to Western blot. Membranes were blotted for PTEN, at two different exposure times. Blot shows two sets of samples from one dissection.

4.3.3 Creation of PTEN-3KR Mutant and Testing with Recombinant, Tagged SUMO

As a tool to study the absence of SUMOylation on PTEN, Dr K Wilkinson constructed GFP-tagged PTEN-WT and PTEN-3KR. This mutant (PTEN-3KR), has the three sites identified as the main/only lysine SUMO acceptor sites on PTEN mutated to arginine, making it non-SUMOylatable (4.3.3.1A.) (Huang *et al*. 2012; Gonzales-Santamaria *et al*. 2012).

The GFP-tagged constructs allowed me to use GFP-trap, which is extremely efficient at immunoprecipitating GFP-tagged proteins under both native and denaturing conditions. It was hoped this would make a useful tool to study PTEN in the context of the absence of SUMOylation, when compared to the WT. This is a novel approach because most previous attempts at assessing the SUMOylation status of PTEN involved use of single or double mutants K289/K266 or K266/K254 (Huang *et al*. 2012; Gonzales-Santamaria *et al*. 2012).

I carried out an IP using GFP trap, after transfection with GFP, GFP-tagged PTEN-WT or PTEN-3KR along with HA-SUMO2 in HEK293T cells (4.3.3.1B and C) and observed that PTEN-WT is significantly more SUMOylated than PTEN-3KR by HA-SUMO2. Although PTEN-3KR is a novel mutant, the results are in line with similar experiments in the literature showing single/double mutation of the same sites reduces PTEN SUMOylation by tagged, recombinant SUMO (Huang *et al*. 2012; Gonzales-Santamaria *et al*. 2012).

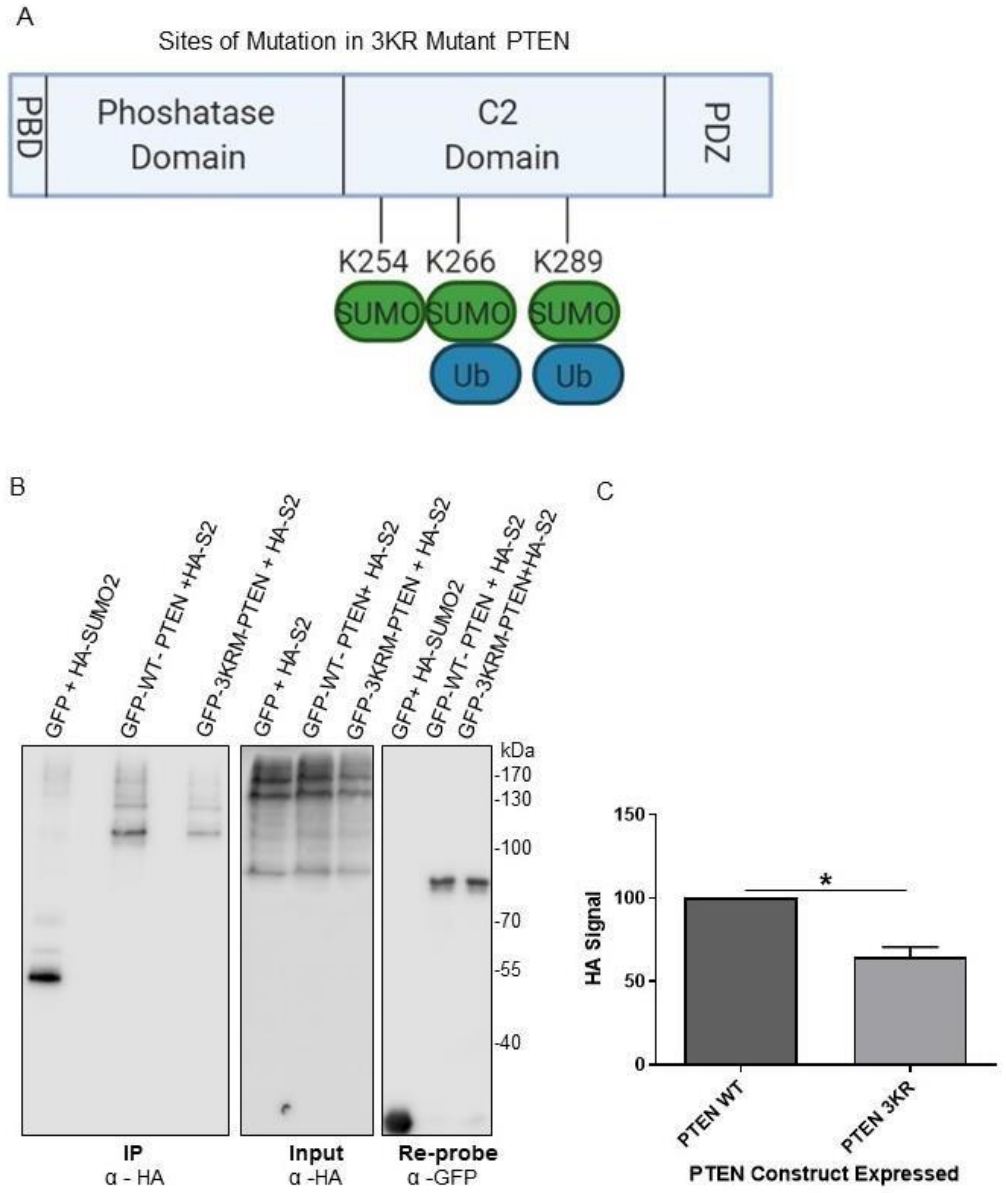

Figure 4.3.3.1. GFP PTEN-3KR is less SUMOylated Compared to WT by HA-SUMO2.

A) Schematic of points of mutation in PTEN-3KR. Schematic showing all identified SUMO sites on PTEN, and which of these are ubiquitinated. Schematic adapted from Xu *et al*. (2014) and CytoskeletonNews; created in Biorender.com. B) Representative blot showing anti-GFP immunoprecipitation of cells transfected with HA-SUMO2, along with either GFP, GFP-WT- PTEN or GFP-3KR-PTEN. 2 days after transfection, cells were lysed in NEM-containing lysis buffer and immunoprecipitated with GFP-Trap. Samples were blotted with HA and GFP antibodies. C) Quantification of B). Immunoprecipitated HA-SUMO2 signal was normalised to both total HA and GFP signal. PTEN-3KR was expressed as a percentage of WT, which was set to a hypothetical value of 100. A one sampled t-test was used to calculate statistical differences ($p=0.0266$; $N=3$).

4.3.4 PTEN-3KR is More SUMOylated than WT by Endogenous SUMO2/3

Most previous attempts at assessing the SUMOylation status of PTEN have involved its expression in cell lines alongside recombinant, tagged SUMO 1 or 2, followed by immunoprecipitation and blotting for tags, or *in vitro* SUMOylation assays (Huang *et al*. 2012; Gonzales-Santamaria *et al*. 2012). In addition to tagged SUMO, I aimed to detect endogenous SUMO, as this would enable me to avoid overexpressing recombinant SUMO in my cells, which can lead to artefacts (Eifler and Vertegaal, 2015). GFP, GFP-tagged PTEN-WT or PTEN-3KR were transfected into HEK239T cells. Cells were immunoprecipitated two days later, and blotted for GFP and endogenous SUMO 2/3. This method revealed a smear above ~65kDa (Figure 4.3.4.1) which we attributed to multiple levels of SUMOylation. Unexpectedly, however, PTEN-3KR was significantly more SUMOylated by endogenous SUMO compared to PTEN-WT (N=6; p=0.0393). This contrasts with Figure 4.3.3.1. and the literature, which suggest mutation of these SUMO sites reduces PTEN-SUMOylation when tagged, recombinant SUMO is used. It is noted that there is a smear on the IP blot at ~30kDa on the GFP lane; it could be that the SUMO antibody is cross reacting with GFP, possibly due to the large amount of GFP immunoprecipitated. This is however not seen when the experiment was later repeated in 2% SDS conditions (Figure 4.3.5.1).

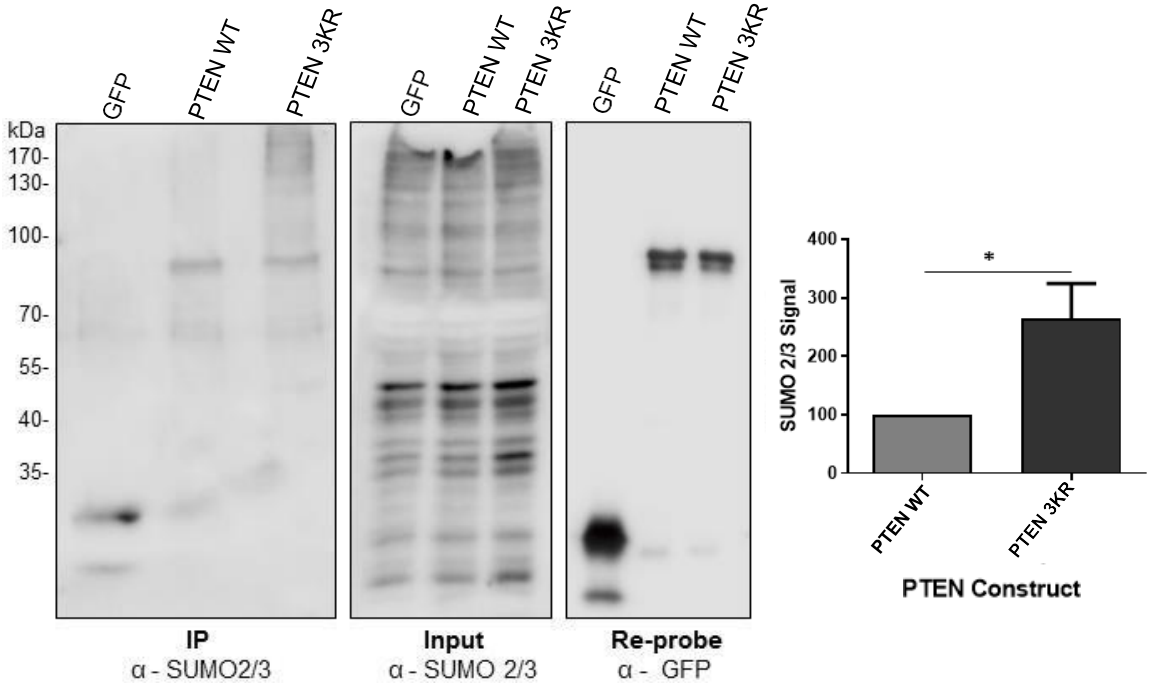

Figure 4.3.4.1. PTEN-3KR is more SUMOylated by Endogenous SUMO2/3 compared to PTEN-WT.
HEK293T cells were transfected with GFP, GFP-tagged PTEN-WT or PTEN-3KR. Two days later, samples were lysed in buffer containing NEM and immunoprecipitated with GFP-Trap before being subject to Western blot and blotted for GFP and SUMO2/3. Representative blot shows endogenous SUMO2/3 modification of PTEN. Graph shows SUMO2/3 signal after being normalised to GFP. PTEN-3KR was expressed as a percentage of WT, which was set to a hypothetical value of 100. A one sampled t-test was used to calculate statistical differences. (N=6; p=0.0393).

4.3.5 Validation of PTEN SUMOylation by Endogenous SUMO in 2% SDS

To further validate the finding that PTEN-3KR is more SUMOylated than WT, and establish if this modification is specific and covalent, immunoprecipitation under harsh denaturing conditions was carried out (Figure 4.3.5.1). Cells were transfected with empty GFP-containing plasmid, or GFP-tagged PTEN-WT or PTEN-3KR, then two days later lysed under denaturing conditions in buffer containing 2% SDS (Rocca, Wilkinson, and Henley 2017). Lysates were then diluted in buffer without SDS before being added to GFP trap beads, so that the final concentration of SDS on in the solution of the beads was <0.5% and IP protocols were completed. The high molecular weight smear was preserved under these conditions, suggesting that the smear is due to specific, direct and covalent modification of PTEN by SUMO, which persists under strong denaturing conditions. It is noted that this is a preliminary result as it was not repeated due to time constraints.

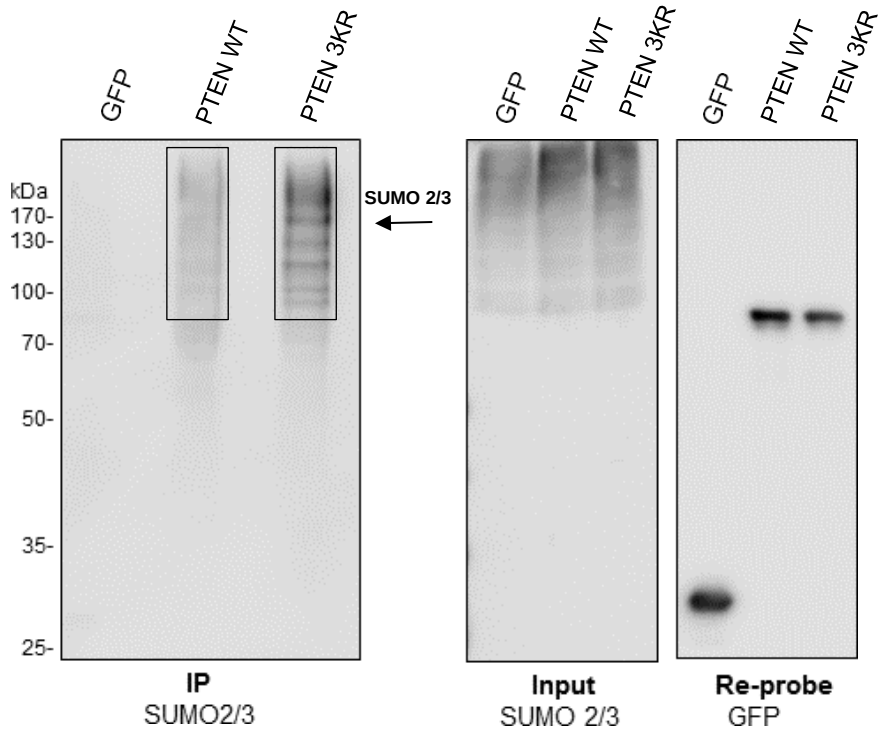

Figure 4.3.5.1. Detection of PTEN modification by SUMO2/3 after cell lysis in 2% SDS

HEK239T Cells were transfected with GFP, GFP-tagged PTEN-WT or PTEN-3KR. The next day cells were lysed in lysis buffer containing NEM and 2% SDS. Lysate was kept at RT for 20 minutes, then centrifuged at RT for 20 minutes. The supernatant was then diluted to 0.5% SDS with lysis buffer without NEM or protease inhibitors before being added to GFP-trap beads, and IP protocol was completed according to Methods 3.4.2).

4.3.6 Detection of Endogenous PTEN SUMOylation- SUMO1

To test if the PTEN-3KR would be more SUMOylated by endogenous SUMO1 as it was for SUMO2/3, GFP immunoprecipitation was carried out as previous, followed by blotting for SUMO1 (Figure 4.3.6.1) Cells were transfected with empty GFP-containing plasmid, or GFP-tagged WT or PTEN-3KR and subject to immunoprecipitation 2 days later. The result shows that PTEN-3KR seems to be modified by SUMO1 more than WT, as was seen with SUMO2/3. This experiment was not repeated as I decided to make SUMO2/3 the focus of my thesis, since AD brains show greater SUMO2/3 dysregulation and minimal SUMO1 dysregulation (Lee *et al*. 2014). Furthermore, SUMO2/3 is shown to colocalise with synaptic marker and AMPAR scaffolding protein PSD-95 to a greater extent than SUMO1, suggesting it is possibly more relevant to regulation of AMPARs (Schnell *et al*. 2002; Colnaghi *et al*. 2019). While interpreted with caution, this preliminary result suggests PTEN-3KR is more SUMOylated than WT by SUMO1, which is consistent with Figure 4.3.4.1. showing PTEN-3KR is more SUMOylated by SUMO2/3.

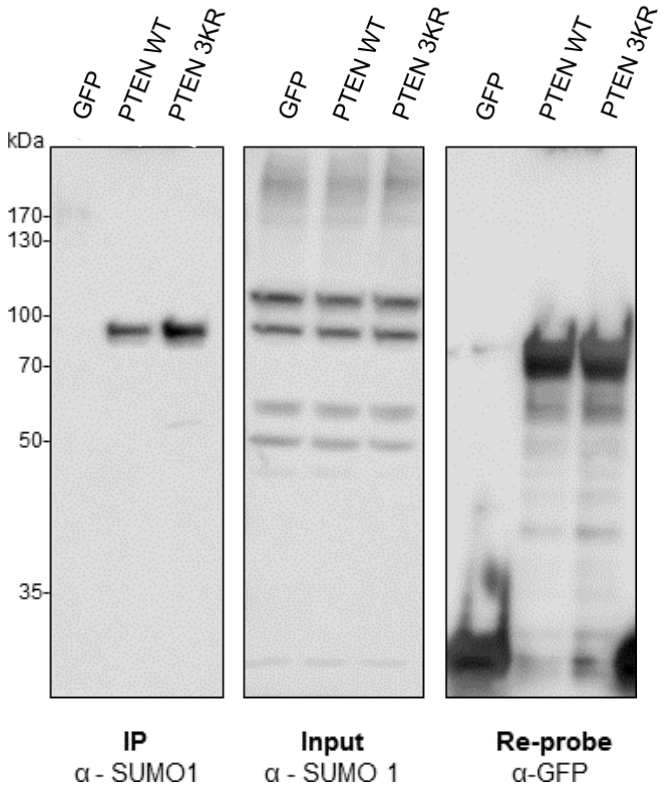

Figure 4.3.6.1. PTEN-3KR is more modified by SUMO1 than PTEN-WT.

HEK293T cells were transfected with GFP, GFP-tagged PTEN-WT or PTEN-3KR. 2 days later, cells were lysed in buffer containing NEM and immunoprecipitation with GFP-Trap was carried out followed by Western blotting for SUMO1 and GFP (N=1).

4.3.7 PTEN-3KR is More Ubiquitinated than WT

Because of the completely unexpected observation that PTEN-3KR is more SUMOylated than WT, I next investigated the ubiquitination status of this mutant (Figure 4.3.7.1). HEK cells were transfected with empty GFP-containing plasmid, or GFP-tagged PTEN-WT or PTEN-3KR and treated with the proteasome inhibitor MG132 24h later (10μM). MG132 was used due to the finding in previous studies that it can aid detection of ubiquitination of PTEN (Gupta and Leslie, 2016). MG123 was added and cells were left for 6 hours, then were immunoprecipitated with GFP- Trap. Subsequent Western blotting for GFP and endogenous ubiquitin showed that PTEN- 3KR is significantly more ubiquitinated than PTEN-WT (N=5; **=p=0.0090). The blot shows a smear which is common for poly-ubiquitinated proteins (Haglund *et al*. 2003). Wu *et al*. (2016) also report double mutation K254R/K266R, or these mutations alone increased the level of PTEN ubiquitination compared to WT. It is not clear exactly why this is seen, although Wang *et al*. (2014) suggest it is due to SUMOylation at K254 and K266 limiting ubiquitination.

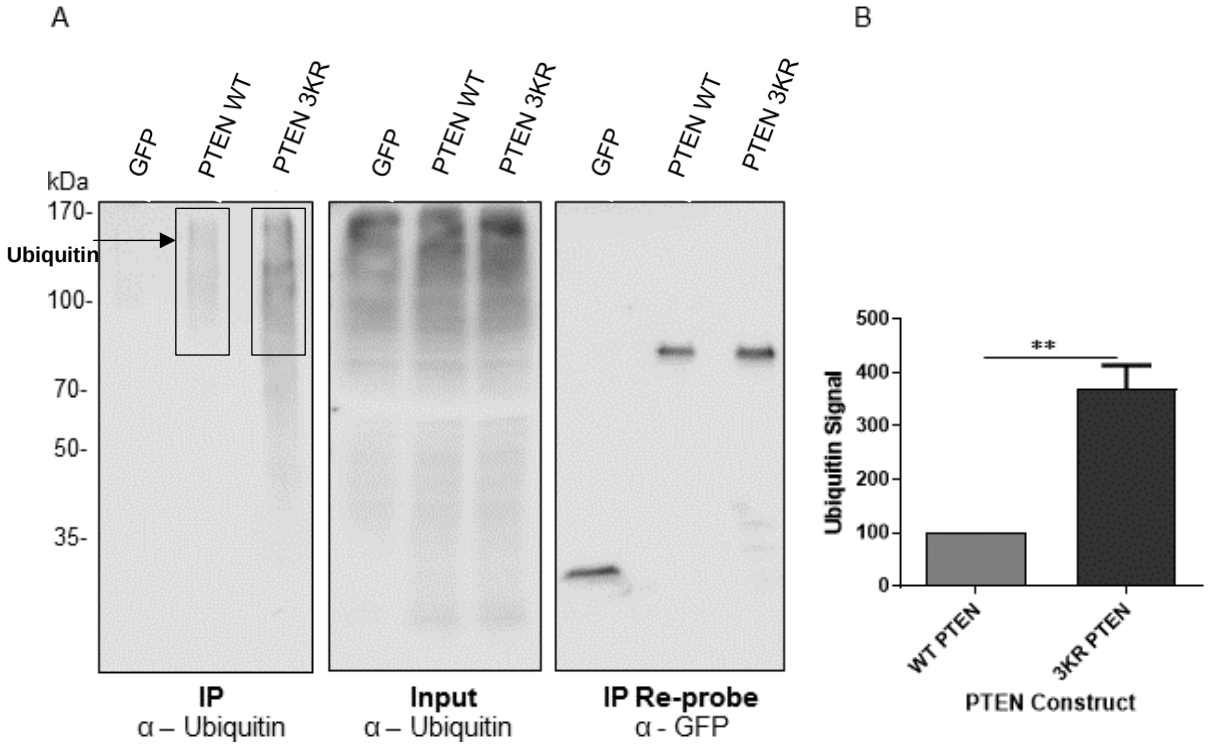

Figure 4.3.7.1. PTEN-3KR is more Ubiquitinated Compared to PTEN-WT.
(A) HEK293T cells were transfected with GFP, PTEN-WT and PTEN-3KR and treated with 10μM MG132 after 24h. 6h later samples were lysed in buffer containing NEM and immunoprecipitated with GFP-Trap. Representative blot shows endogenous ubiquitin modification of PTEN. (B) Quantification of (A). PTEN-3KR was expressed as a percentage of WT, which was set to a hypothetical value of 100. A one sampled t-test was used to calculate statistical differences. (N=5; **=p=0.0090).

4.3.8 SENP treatment cleaves SUMO2/3 Chains Without Effecting Ubiquitin

As explained in Section 4.1.3, previous work has suggested SUMO and ubiquitin can form hybrid chains on target proteins (Tatham *et al*. 2008). To explore whether hybrid chains can form on PTEN, immunoprecipitation was carried out, followed by treatment of the immunoprecipitated proteins with the recombinant catalytic domain of human SENP1 enzyme. This enzyme will remove SUMO, but not ubiquitin, from the immunoprecipitated proteins and has been used previously in our lab by Dr Richard Seager, who reported SUMO1 and 2 on Mitochondrial fission factor (MFF) were entirely removed by SENP1. This enzyme has also been used successfully by Bekes *et al*. (2011) to remove SUMO chains. It was hoped that using this enzyme could reveal whether hybrid SUMO-ubiquitin chains are forming (since removal of SUMO would be expected to lead to reduction of ubiquitination). It would also help clarify whether one modification is recruiting or obstructing the other.

The SENP enzyme entirely cleaved SUMO from PTEN, while having no effect on ubiquitin (Figure 4.3.8.1). This preliminary result was not repeated due to time constraints and therefore should be interpreted with caution, however it suggests that it is possible that SUMO forms chains on ubiquitin on PTEN, but ubiquitin does not form chains on SUMO. Although the PTEN-3KR was more SUMOylated and ubiquitinated than PTEN-WT as seen previously, the interaction between these modifiers in this context after SENP treatment does not seen to vary between PTEN-WT and PTEN-3KR. The result suggests that under these conditions, SUMO on PTEN is not recruiting a STUBL. Furthermore, this experiment helps to validate that SUMO2/3 antibody is specifically detecting SUMO2/3 modification of PTEN.

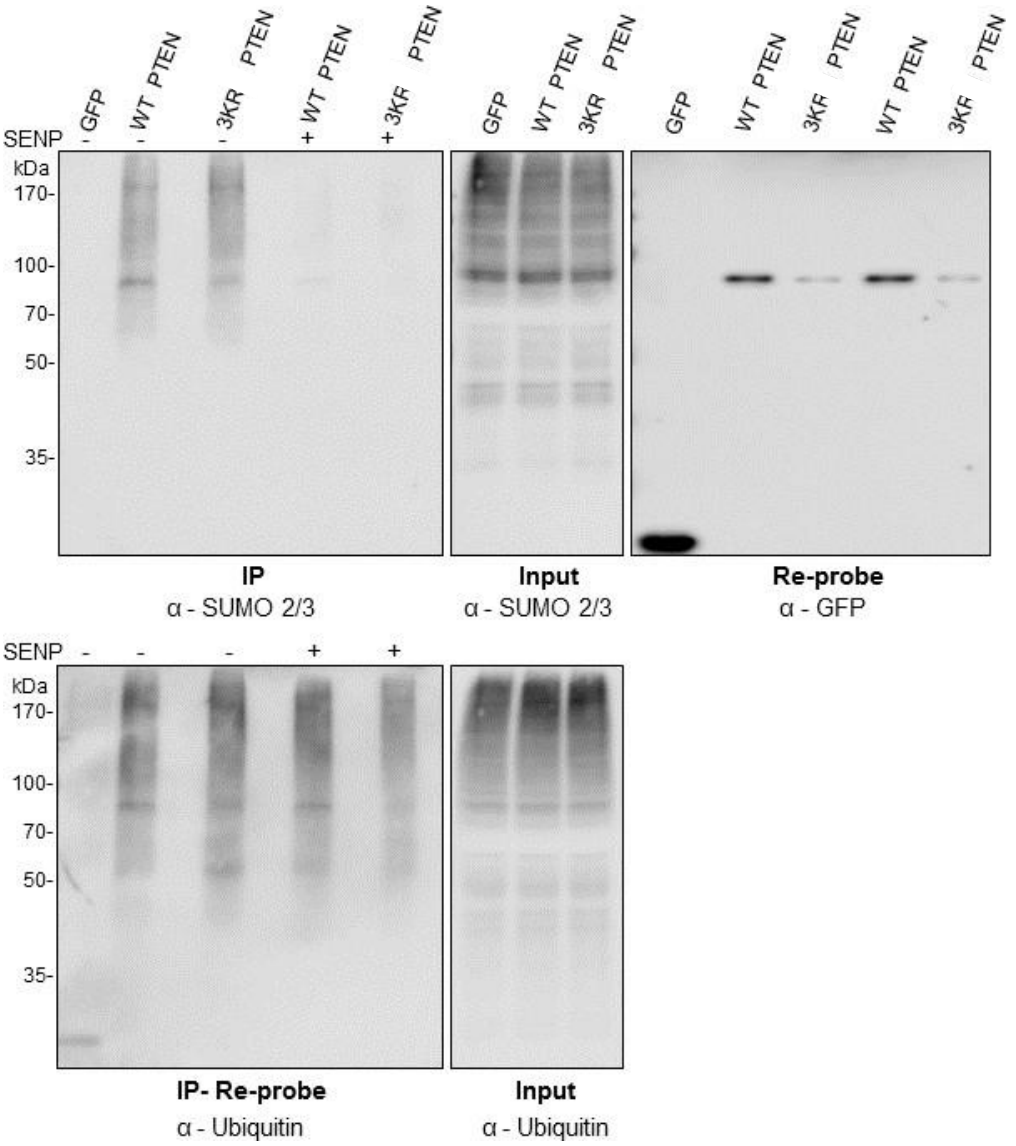

Figure 4.3.8.1. SENP1 treatment removes SUMO2/3, but not ubiquitin from PTEN.

HEK293T cells were transfected with GFP, GFP-WT-PTEN or GFP-3KR-PTEN. 3 days later, cells were lysed in buffer containing NEM and immunoprecipitated on GFP-Trap beads. Beads were then washed and 100nM recombinant SENP1 was then added to one set of tubes of beads. All samples were then incubated for 1 hour at 37°C, 4x sample buffer was added and samples were boiled at 95°C for 10 minutes. Samples were then Western blotted for SUMO 2/3, then stripped and reblotted for Ubiquitin (N=1).

4.3.9 Testing SUMO/Ubiquitin Interplay *with SUMO and Ubiquitin Overexpression*

To further investigate SUMO/ubiquitin crosstalk, experiments were carried out to establish whether over-expression of each protein could alter PTEN modification by the other. To this end, PTEN-WT was immunoprecipitated in HEK cells, with or without expression of HA-SUMO2 (Figure 4.3.9.1.A) or HA-ubiquitin (Figure 4.3.9.1.B), and ubiquitin and SUMO2/3 were blotted for in each case. Over-expression of HA-SUMO2 seemed to reduce PTEN ubiquitination but this did not reach significance (Figure 4.3.9.1.A; N=3, p=0.0541), and over-expression of HA-ubiquitin did not alter PTEN SUMOylation (Figure 4.3.9.1.B) (N=3). Ubiquitination was reduced by SUMO2 expression in all three repeats in Figure 4.3.9.1.A, but the extent to which it was reduced varied substantially (85%, 35% and 69%), so this is likely why this result did not reach significance. Nonetheless, with this in mind, it is therefore likely that HA-SUMO2 expression reduces ubiquitination of PTEN; however, clearly more repeats would improve confidence in this result.

These data indicate that SUMO and ubiquitin may be competing for the same sites on PTEN as suggested by Gonzales-Santamaria *et al*. (2012). This also supports observations made by Wang *et al*. (2014), who report that SUMOylation at K266 thought to limit ubiquitination, however, it is not clear whether SUMOylation may also sterically block ubiquitination at other sites. Nonetheless, according to this result, if SUMO is overexpressed and there is more SUMO modification of PTEN, ubiquitination is reduced.

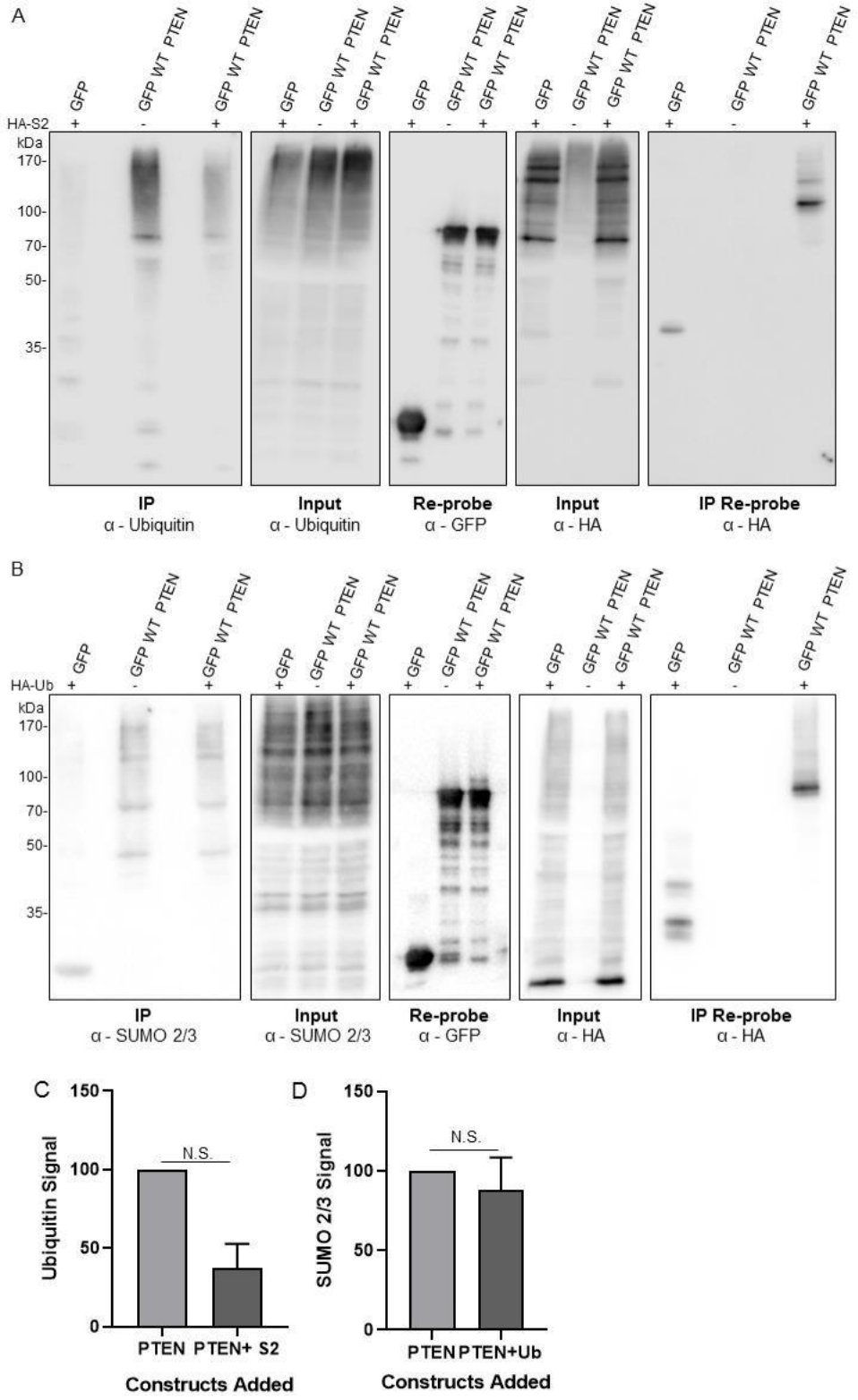

Figure 4.3.9.1. Effect of Overexpression of SUMO2 on PTEN ubiquitination and overexpression of ubiquitin on PTEN SUMOylation.

(A) HEK293T cells were transfected with GFP+ HA-SUMO2, GFP-WT-PTEN or GFP-WT-PTEN + HA-SUMO2 (S2). Two days later, cells were lysed in buffer containing NEM and subject to immunoprecipitation (IP). Samples were blotted for ubiquitin, HA and GFP. (B) HEK cells were transfected with GFP+ HA-ubiquitin (HA-Ub), GFP-WT-PTEN or GFP-WT-PTEN + HA-Ub. 2 days later, cells were lysed and subject to IP. Samples were blotted for SUMO2/3, HA and GFP. C) Quantification of ubiquitin signal in A) after being normalised to GFP. PTEN-3KR was expressed as a percentage of WT, which was set to a hypothetical value of 100, and analysed in a one-sample t-test (N=3**;** p=0.0541). D) SUMO2/3 signal was analysed as in C) (N=3).

4.4 Discussion

4.4.1 Detection of SUMO and Ubiquitin on PTEN

Here I show that I can detect both recombinant and endogenous SUMOylation of PTEN in GFP-Trap immunoprecipitation experiments by using GFP-tagged PTEN (Figures 4.3.3.1-4.3.4.1.). Initial blotting of PTEN suggested the higher band of PTEN shown in Figure 4.3.2.1. could be SUMOylated PTEN, consistent with data from Bassi *et al*. (2013), however, subsequent literature investigation showed that this band was also seen by Hopkins *et al*. (2013), who suggest it is a different, longer isoform of PTEN which they named “PTEN-L”, and therefore not post-translationally modified PTEN. Thus, blotting for this upper band to detect PTEN PTMs may not be valid, and optimising the immunoprecipitation method was essential. The creation of GFP-tagged PTEN also made detection of endogenous ubiquitin possible.

4.4.2 Differences Between Recombinant and Endogenous SUMO on PTEN

In Figure 4.3.3.1 I show that in my hands, PTEN-3KR is less SUMOylated by HA-tagged SUMO2, compared to WT, which fits with the literature regarding single/ double PTEN mutants. However, the evidence in this thesis suggests that endogenous SUMO may behave differently, and PTEN-3KR exhibits enhanced SUMOylation compared to WT. My preliminary result indicating that the endogenous SUMO-PTEN modification persists under 2% SDS conditions (Figure 4.3.5.1.), goes towards validating that interaction detected is specific and covalent (Rocca *et al*. 2017).

It is not clear exactly why PTEN-3KR is less SUMOylated by tagged, recombinant SUMO and more SUMOylated by endogenous SUMO compared to WT. This change in modification site can occur with ubiquitination; some studies have shown ubiquitination is increased when certain ubiquitination sites are mutated (Wang *et al*. 2014; Wu *et al*. 2016). The additional endogenous SUMOylation of PTEN-3KR could occur because SUMO preferentially conjugates at different sites when known SUMO sites are mutated, for example K102 is also suggested as a possible SUMO site according to SUMOplot software (Gonzales-Santamaria *et al*. 2012). Evidence for this comes from Wang *et al*. (2014), who suggest SUMOylation at one site may block SUMOylation at another site given the large size of SUMO (>90 residues), the lack of band shift between single and double mutants, and the proximity between the K254 and K266 sites. Furthermore, mutations in PTEN-3KR may increase binding at a SIM site, possibly because SUMO cannot bind at sites in close proximity due to its size (Huang *et al*. 2012). Additional SUMO binding at SIMs can move SUMO into closer proximity with additional SUMO sites (Wang and Dasso 2009); this may enhance

SUMOylation of PTEN-3KR at these SUMO sites. Positions 98-101 and 317-320 are suggested SIM sites on PTEN which could non-covalently bind SUMO (Bawa-Khalfe *et al*. 2016).

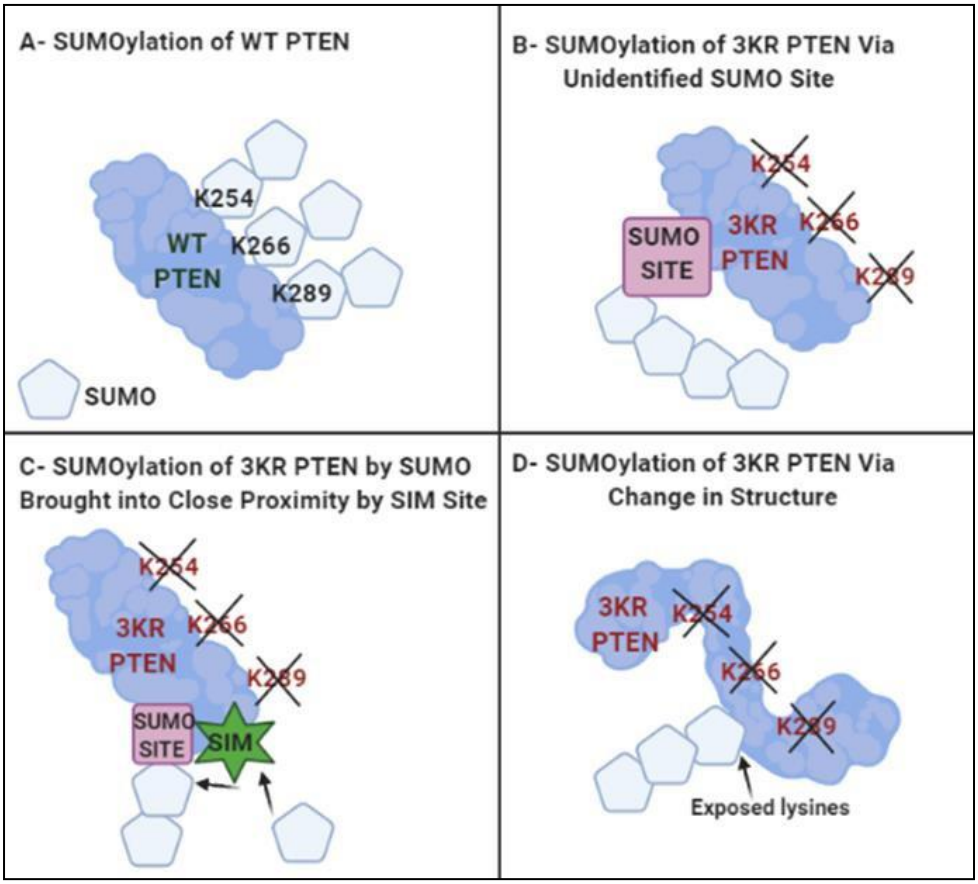

Figure 4.4.2.1. Schematic Depicting Potential Reasons for Enhanced SUMOylation of PTEN-3KR.
PTEN-WT is SUMOylated at K254, K266 and K289 (Huang *et al. 2012;* Santamaria *et al*., 2012). B) Enhanced SUMO on PTEN-3KR could be attaching to an additional SUMO site; K102 is a suggested possible SUMO site on PTEN (Gonzales- Santamaria *et al*., 2012). Furthermore, SUMOylation at one site may block SUMOylation at another (Wang *et al*., 2014; Huang *et al*., 2012), so mutations in PTEN-3KR may free up more lysines to be SUMOylated. C) SIM sites on PTEN have been identified by Bawa- Khalfe *et al*. (2016), mutations in PTEN-3KR may enhance SUMO binding at SIM sites. SUMO binding at SIMs can bring SUMO closer to additional SUMO sites which it could SUMOylate (Wang and Dasso, 2009). D) PTEN-3KR could be in a different structure to WT; it could be that SUMO-site mutations induce this change, leaving more potential SUMO site lysines exposed for SUMO to modify. Huang *et al*. (2016) tested K266A, K266R and K266Q PTEN mutants in molecular dynamic simulations and found little effect on PTEN conformation, but PTEN-3KR has not been tested in this way. Schematic based on work by Bawa-Khalfe *et al*. (2016); Huang *et al*. (2012); Gonzalez-Santamaria *et al*. (2012). Made in Biorender.com with premade shapes.

There is also a possibility that the mutations could cause PTEN to be in a different conformation which could potentially leave more SUMOylatable lysines exposed for SUMO to bind to (Huang *et al*. 2016). However, Huang *et al*. (2016) examined this and found the single mutants K266R, K266A and K266Q had little effect on PTEN structure in molecular

dynamics simulations, although double mutants and PTEN-3KR have not been tested in these experiments. It is also noted that SUMO modification of PTEN could change its conformation; Gonzales-Santamaria *et al*. (2012) suggest that SUMO binding to PTEN can block intramolecular interactions that enable it to form the closed conformation, thus forcing it into the open conformation. Although it was not expected to have enhanced SUMOylation, I nonetheless reasoned that PTEN-3KR can still be used as a tool to study the effects of enhanced SUMOylation/ ubiquitination of PTEN.

4.4.3 Overexpression of SUMO may Confound Results

Consistent with the literature, when I examined tagged, recombinant SUMO conjugation to my WT and PTEN-3KR constructs, I saw a significant reduction in HA-SUMO2 on PTEN-3KR compared to WT (Figure 4.3.3.1.). These data are in agreement with studies using similar SUMO overexpression techniques, showing single and double mutations of these sites reduced SUMOylation (Huang *et al*. 2012; Gonzales-Santamaria *et al*. 2012). Crucially, however, these results with tagged SUMO differ to my results with endogenous SUMO (Figure 4.3.4.1.).

Previous studies have mostly looked at tagged and/or recombinant SUMO in cells, or in *in vitro* SUMOylation assays, rather than endogenous SUMO; Eifler and Vertegaal (2015) note that compared to endogenous, exogenous SUMO expression can increase levels of SUMOylation of target proteins and can lead to over-expression artefacts if it is not controlled. Therefore, I argue that my experiments with endogenous SUMO are more physiologically relevant due to less confounding factors from SUMO overexpression. Interestingly, in Figure 4.3.9.1., HA-SUMO2 overexpression seemingly led to a reduction of PTEN ubiquitination (although this did not quite reach significance). This suggests again that recombinant SUMO may have different effects. This may be because SUMO overexpression can enhance target SUMOylation (Eifler and Vertegaal, 2015) so there could be more SUMO to displace ubiquitin on PTEN.

4.4.4 Discrepancies Regarding "Non-SUMOylatable" Mutants

The only study to date which tested endogenous SUMOylation of PTEN by Bawa-Khalfe *et al*. (2016), showed that the double mutant K266A/K254A was less modified than WT by SUMO1. Although it is noted the PTEN-3KR mutant contains an additional site of mutation to this mutant, and lysines in PTEN-3KR were mutated to arginine rather than alanine, this is in contrast to my preliminary data on SUMO1 (Figure 4.3.6) showing that PTEN-3KR is more SUMOylated by SUMO1. Furthermore, Bawa-Khalfe *et al*. (2016) failed to detect endogenous SUMO2 or 3 on PTEN-WT, while I have repeatedly shown this in this chapter.

This suggests that my system may be more sensitive to detecting SUMOylation, given that other groups have provided evidence that PTEN is SUMOylated by recombinant SUMO2 (Gonzales- Santamaria *et al*. 2012). Furthermore, Bawa-Khalfe *et al*. (2016) also showed the K266A/K254A mutant was less ubiquitinated, which also goes against my own observations with PTEN-3KR (Figure 4.3.7.). Similarly, Wu *et al*. (2016) and Wang *et al*. (2014) both showed that PTEN K266R/K254R mutant is actually more ubiquitinated than WT.

It is noted that Huang's work focuses on SUMO1 and this thesis focuses on SUMO2/3. It could be argued that these proteins conjugate differently, however, I have preliminary data showing PTEN-3KR is also more SUMOylated by SUMO1 than WT (Figure 4.3.6.1), as it is by SUMO 2/3 (Figure 4.3.4.1). I have also shown that in my samples, endogenous SUMO2/3 acts differently to recombinant SUMO2, which conjugates more to WT than PTEN-3KR; the literature also shows mutation of residues K254, K266 or K289 sites reduces modification by recombinant SUMO2 as well as SUMO1 (Gonzales-Santamaria *et al*. 2012). Therefore, it seems more likely that the differences in conjugation are due to whether SUMO is endogenous or expressed, rather than due to paralogue specific effects.

4.4.5 PTEN Ubiquitination

Regarding ubiquitination, as explained, it initially seems surprising that the PTEN-3KR is also more ubiquitinated (Figure 4.3.7.1), given that K266 and K289 are ubiquitinated and these are mutated in PTEN-3KR (Gupta and Leslie, 2016, Trotman *et al*. 2007). However, there are additional known ubiquitin sites not mutated in the PTEN-3KR, such as K13 (Trotman *et al*. 2007). Furthermore, Wang *et al*. (2014) showed single mutations of K254R and K266R did not alter PTEN ubiquitination level, but mutations of both sites simultaneously increased PTEN ubiquitination. This was suggested to be because SUMOylation at these sites blocks ubiquitination (Wang *et al*. 2014). Similarly, Wu *et al*. (2016) also showed K266R mutation had a small effect in increasing ubiquitination, but K254R or double mutant hugely increased ubiquitination. There is some controversy in this area as Bawa-Khalfe *et al*. (2016) found K266A/K254A double mutant to be less ubiquitinated, although PC-3 cells were used, which have homozygous deletion of PTEN (Vlietstra *et al*. 1998). Therefore, although in some ways unexpected, my results are generally in line with the literature. It is possible that blocking SUMOylation at sites mutated in PTEN-3KR (K254 and K266) causes the increased ubiquitination as suggested by Wang *et al*. (2014).

4.4.6 SUMO/Ubiquitin Crosstalk- SENP/Ubiquitin Overexpression

My data suggests that SUMO and ubiquitin modification are increased on PTEN-3KR compared to WT (Figures 4.3.4.1 and 4.3.7.1), and increasing PTEN SUMOylation reduces ubiquitination on PTEN-WT (although it is noted it did not quite reach significance) (Figure 4.3.9.1 Some possible explanations for both of these effects involve crosstalk.

Although it has been shown that PTEN SUMOylation can promote ubiquitination of PTEN through increasing association with ubiquitin ligase WWP2 (Bawa-Khalfe *et al*. 2016), overall, SUMO seems to negatively regulate ubiquitin modification of PTEN (Gonzales-Santamaria *et al*. 2012; Wang *et al*. 2014, Wu *et al*. 2016). My results show that increasing HA-ubiquitin conjugation to PTEN does not affect modification by SUMO2/3, but increasing HA-SUMO2 conjugation reduces ubiquitin modification (Figure 4.3.9.1). This is in line with Gonzales-Santamaria *et al*. (2012), who found that SUMO1 conjugation reduces mono-ubiquitination, but mono-ubiquitination does not reduce SUMO1 conjugation. They also support Wang *et al*. (2014), who found SUMOylation at K254 and K266 can limit ubiquitination, as well as Wu *et al*. (2016). While not well characterised, the protein RWD-containing Sumoylation Enhancer (RSUME), can increase SUMOylation of targets including PTEN (Wu *et al*. 2016; Carbia-Nagashima *et al*. 2007). This increased SUMOylation through RSUME has an effect of reducing PTEN ubiquitination (Wu *et al*. 2016). Mutation of both K254R and K266R SUMO sites blocked the effect of RSUME on inhibiting PTEN ubiquitination, while individual mutation of each site had no effect, suggest both sites are critical in RSUME-mediated PTEN ubiquitination inhibition (Wu *et al*. 2016). PTEN-WT stability was increased by RSUME overexpression, but stability of K254R/K266R double mutant was not affected. (Wu *et al*. 2016). It was concluded that by increasing SUMOylation, RSUME reduces ubiquitination on PTEN, which increases its stability (Wu *et al*. 2016). Therefore, my results are in line with the literature, as increasing PTEN modification by SUMO2/3 decreased ubiquitination. My data argues against the findings that SUMO on PTEN recruits a STUbL.

SUMO and ubiquitin may compete for the same sites (Gonzales-Santamaria *et al*. 2012), as K266 and K289 are both SUMOylated and ubiquitinated (Huang *et al*. 2012; Gonzales-Santamaria *et al*. 2012; Bassi *et al*. 2013; Trotman *et al*. 2007; Gupta and Leslie, 2016), and SUMOylation at some sites may block ubiquitination at other sites. Evidence for this comes from Wang *et al*. (2014), who found SUMOylation at K254 and K266 sites limits ubiquitination (Wang *et al*. 2014). They suggest that SUMOylation at one site may block SUMOylation at another site given the lack of band shift between single and double mutants, and the proximity between K254 and K266 sites. Therefore, considering ubiquitin is a similar size (>70 residues), it is possible SUMO could also block ubiquitination at another site; indeed, Wang *et al*. (2014) suggest that SUMOylation of PTEN at K254 and K266 is necessary to limit ubiquitination level and stability, as mutation of these sites increases ubiquitination.

Moreover, concurrent mutation of K254R and K266R, or K254R alone increases ubiquitination, and reduces SUMOylation (Wu *et al*. 2016). This suggests SUMOylation at K254 may block ubiquitination at other sites, especially given that this paper also shows K254 to be the dominant SUMOylation site, and its proximity to the K266 site. However, this does not explain why/ how the PTEN-3KR mutant is also more SUMOylated. It is also noted that these results are not necessarily physiologically relevant, as they involved overexpression of tagged SUMO and ubiquitin constructs; it may be that endogenous SUMO in cells is not enough to displace ubiquitin from PTEN.

4.4.7 SUMO/Ubiquitin Crosstalk- SENP Enzyme Treatment

Figure 4.3.8 involved removal of SUMO from PTEN using SENP enzyme treatment (an effect seen previously on other proteins by Dr R Seager in the lab). While interpreted with caution due to the lack of repeats, the SENP treatment removed SUMO on PTEN, without effecting PTEN ubiquitination (Figure 4.3.8.1), suggesting that ubiquitin is not attaching to PTEN via SUMO. This contrasts with Bawa-Khalfe *et al*. (2016) who found SENP1 expression removed both SUMO and ubiquitin from PTEN, however there are methodological differences in terms of modulation of protein expression vs enzyme application, and cell types used. It also contrasts with Tatham *et al*. (2008) who suggest SUMO2/3 and ubiquitin can form mixed chains, although it is noted that they were not examining PTEN. This result has also gone towards validating my SUMO and ubiquitin antibodies and ruling out cross reactivity between them.

4.5 Conclusion

In conclusion, in this chapter I have gone some way towards addressing gaps of knowledge in PTEN-SUMOylation by optimising endogenous PTEN SUMOylation and ubiquitination detection, creating a tool which can be used to study the enhanced SUMOylation of PTEN, and providing evidence that mutants previously thought to be non/reduced SUMOylated may actually be more SUMOylated than WT when endogenous SUMO is concerned. I also have initial data which helps to validate the endogenous detection of SUMOylation of PTEN in 2% SDS. I provided evidence that PTEN-3KR is more SUMOylated by endogenous SUMO2/3, and have preliminary evidence that the same is true for SUMO1.

The question of whether PTEN SUMO-site mutants are more or less SUMOylated than WT is important given the conclusions drawn from previous work on these mutants in the literature. SUMOylation is suggested to control PTEN localisation and ability to regulate Akt pathway (Huang *et al*. 2012), characteristics both likely to be relevant to PTEN function in neurons

and plasticity (Jurado *et al*. 2010; Liu *et al*. 2018). It has been suggested that SUMOylation at specific sites on PTEN can block SUMOylation at another site; Huang *et al*. (2012) suggest concomitant SUMOylation of K254 and K266 cannot occur due to the relatively large space needed for SUMO modification. Therefore, it may be that blocking SUMOylation through mutation of K254, K266 and/or K289 enables more SUMO to bind at other sites on PTEN-3KR, leading to enhanced SUMOylation. This could also possibly occur through a SIM site on PTEN (Bawa-Khalfe *et al*. 2016).

Additionally, I demonstrated that PTEN-3KR is more ubiquitinated than WT, which is largely fitting with the literature on double mutants (Wang *et al*. 2014; Wu *et al*. 2016). I have provided further evidence of the order of SUMOylation and ubiquitination by demonstrating that SUMOylation is likely to be able to limit ubiquitination of PTEN, similar to effects seen by Wang *et al*. (2014) who showed that SUMOylation is needed to limit ubiquitination. Using the SENP1, enzyme, I have preliminary data suggesting that ubiquitin is not forming chains on SUMO on PTEN, but more work is needed to establish whether SUMO is forming chains on ubiquitin, which could be carried out using a de-ubiquitinating enzyme in similar *in vitro* ubiquitination assays.

Clearly more research is needed to establish the exact role of SUMO and ubiquitin in terms of how they may regulate each other, and how they may influence localisation, as there is controversy about this (Bassi *et al*. 2013; Huang *et al*. 2012; Bawa-Khalfe *et al*. 2016). A major issue in making conclusions about the current literature is that there is little homogeneity between studies in terms of cell types and methods used.

4.6 Future Directions

To further these results, the following experiments should be conducted

- Mutate the SIM site in PTEN-3KR and repeat immunoprecipitation to detect SUMOylation, to establish whether SUMO on PTEN-3KR is enhanced by SIM-sites
- To clarify whether SUMO is forming chains on ubiquitin, experiment in Figure 4.3.8 could be repeated with a DUB such as Ubiquitin-Specific Protease-2a (USP2a) which would remove ubiquitin from PTEN (Graner *et al*. 2004).
- Immunoprecipitation of PTEN-3KR to establish whether it is in a different conformation to WT, using a methodology developed by Odriozola *et al*. (2007) and also used by (Rahdar *et al*. 2009). This involves immunoprecipitating tagged mutants of the PTEN C-tail and the rest of WT or PTEN-3KR, and immunoprecipitating the C-tail with the other fragment. If PTEN-3KR was able to immunoprecipitate more C-tail

fragment than WT, this would imply it occurs more in the “closed” conformation, due to reduced ability to form intramolecular interactions between the C-tail and PTEN-3KR. This experiment would in part test the theory by Gonzales-Santamaria (2012) that SUMOylation can force PTEN into the “open” conformation through blocking C-tail/C2 domain interactions.

- Repeat immunoprecipitation for SUMO and ubiquitin on PTEN in neurons to establish if PTEN is SUMOylated in neurons, and if PTEN-3KR is more SUMOylated as it is in HEK293T cells.

Results Chapter 2

Characterising PTEN-3KR in Cell Lines and Neurons

5 Characterising PTEN-3KR in Cell Lines and Neurons

5.1 Introduction

5.1.1 PTEN Functions and Post-Translational Modifications

In this chapter, I aimed to characterize PTEN-3KR in terms of its stability, localization, and function, as previous work has shown SUMOylation of PTEN can regulate these properties (Wang *et al*. 2014; Huang *et al*. 2012). This PTEN regulation via SUMOylation may have implications in neurons, as the phosphatase activity of PTEN is critical in NMDAR-dependent LTD, and can drive depression of AMPAR-mediated neurotransmission (Jurado *et al*. 2010). SUMOylation can regulate memory consolidation and plasticity, and SUMOylation dysregulation is present in the Tg2576 AD mouse models and *post-mortem* AD brains (Lee *et al*. 2014). Furthermore, ubiquitin-mediated PTEN degradation has been suggested to underlie the PTEN loss seen in AD (Kwak *et al*. 2010), highlighting the importance of post-translational regulation of PTEN stability in neurons. Therefore, a point of interest is PTEN SUMOylation in neurons, which has not yet been assessed.

Previous work has tested single/ double PTEN mutants including K266A/K289A, K254A/K266A and K254R/K266R, with reduced SUMOylation capacity (Huang *et al*. 2012; Gonzalez-Santamaria *et al*. 2012; Wang *et al*. 2014; Bawa-Khalfe *et al*. 2016). Here I test the novel PTEN-3KR mutant PTEN, which contrary to our expectations, displays enhanced ubiquitination and SUMOylation by endogenous SUMO/ubiquitin compared to WT. Analysis of this mutant will avoid potential confounding variables in previous studies such as SUMO overexpression artefacts (Eifler and Vertegaal; 2015), and modulation of global SUMOylation which can confound results due to the large range of SUMO targets related to PTEN including Akt (de la Cruz-Herrera *et al*. 2015). The aim of this chapter is therefore to test how enhanced SUMOylation and ubiquitination of PTEN can affect PTEN regulation, and if this has a role in the context of AMPAR trafficking.

5.1.2 Role of PTEN SUMOylation in Membrane Localization

There is debate regarding the role of SUMOylation on the localization of PTEN, and studies have separately examined effects of SUMOylation at different sites. PTEN localization is an important issue as its membrane localisation is critical in Akt pathway regulation and tumorigenesis (Rahdar *et al*. 2009; Vazquez *et al*. 2006; Huang *et al*. 2012). Huang *et al*. (2012) initially reported that in subcellular fractionation experiments in HEK293T cells, PTEN K266R was almost totally absent from the membrane fraction compared to WT, and the K254R

mutant was also slightly reduced in the membrane fraction. They concluded that addition of positively charged SUMO at K266 on PTEN facilitates membrane localization, through increasing electrostatic interactions between PTEN and the electronegative membrane (Huang *et al*. 2012).

Gonzales-Santamaria *et al*. (2012) also reported reduced-SUMOylation mutants K289A, K266A and double mutant K289A/K266A exhibit increased cytoplasmic localisation in PC-3 and MCF-7 cells compared to WT (although this confocal microscopy experiment compared nuclear to cytoplasmic localisation; membrane localisation was not quantified). Further experiments involved Vesicular Stomatitis Virus (VSV) infection to test PTEN's role in antiviral response in MEFs, which showed that VSV causes PTEN to move to the membrane, where it co-localises with SUMO1 or 2. Gonzales-Santamaria *et al*. (2012) hypothesise that membrane localisation and SUMOylation may be associated and suggest SUMO may block PTEN C-terminus interactions with the C2 domain, forcing the protein into an "open" conformation, which facilitates membrane localisation. This differs from the view of Huang *et al*. (2012), who suggest PTEN SUMOylation aids membrane localisation independently of a conformational change.

In neurons, the localisation of PTEN may also be important in plasticity; PSD-95 interactions with PTEN are critical in LTD, possibly by allowing PTEN to position near the membrane and improving its catalytic activity (Jurado *et al*. 2010) (See 5.1.7 for more information on PTEN and AMPAR trafficking).

5.1.3 Other Effects of PTEN SUMOylation on Localization

Bassi *et al*. (2013) reported SUMOylation at K254 facilitates nuclear localisation of PTEN, since a reduced-SUMOylatable K254R mutant failed to be retained in the nucleus in HEK293T cells in imaging experiments. This was contrasted by Bawa-Khalfe *et al*. (2016) who showed by subcellular fractionation that a PTEN K254A/K266A double mutant was localised more in the nucleus rather than cytosolic fraction in human prostate adenocarcinoma (LNCap) cells (Bawa-Khalfe *et al*. 2016). LNCap cells contain a frame-shift PTEN mutation and do not express PTEN (Vlietstra *et al*. 1998), and were presumably used in this experiment so that a tagged PTEN could be observed without confounding effects of endogenous PTEN. These contrasting results could be due to differences in cell types used, also, Bawa-Khalfe did not specifically test the location of K254A/K266A mutant in HEK293T cells as Bassi *et al*. (2013) did. The mutants in these studies also varied in terms of amino acid substitution and single versus double mutants. Different methods were used to analyse PTEN mutant localisation; Bassi *et al*. used confocal imagining while Bawa-Khalfe *et al*. used subcellular fractionation.

In summary, it seems there is little consistency between results on effects of PTEN -

SUMOylation on localisation, which may be due to differences, in methods, mutants and cell

types used. Many studies to date have relied on methods such as overexpressing recombinant SUMO, or manipulating global SUMOylation to asses PTEN SUMOylation; testing endogenous PTEN SUMOylation may be more valid (Eifler and Vertegaal; 2015). In neurons, PTEN is redistributed to the post synaptic density after NMDAR stimulation, where it co-localises with PSD-95; a process critical in LTD (Jurado *et al*. 2010). Therefore, if SUMOylation of PTEN in neurons can regulate localisation, this may have the potential to influence plasticity.

5.1.4 Effects of PTEN SUMOylation on the Akt Pathway

PIP_3 and its regulation through PTEN phosphatase activity are important in plasticity; PTEN can counter the PIP_3 increase in spines after NMDAR-LTD, and PIP_3 levels control AMPAR surface expression (Jurado *et al*. 2017; Arendt *et al*. 2010; Arendt *et al*. 2014). Furthermore, PTEN's phosphatase activity is critical in NMDAR-LTD and can influence AMPAR transmission (Jurado *et al*. 2010). Furthermore, through effects on the Akt pathway, PTEN attenuation can relieve plasticity deficits and restore AMPAR and NMDAR levels in PTSD mouse models (Liu *et al*. 2018).

PTEN SUMOylation has not yet been tested in neurons, but Huang *et al*. (2012) report that SUMO plays a role in PTEN's ability to limit PIP_3 levels and Akt signalling in cell lines. They examined the signalling capability of PTEN-WT and reduced-SUMOylation mutants using PC-3 cells, which contain a homozygous PTEN exon deletion (Vlietstra *et al*. 1998). Injection of immunodeficient mice with PC-3 cells resulted in tumour formation, and expression of PTEN-WT in these cells was able to suppress tumour growth shown by bioluminescent imaging of tumours. However, K266R and K254R PTEN mutants were unable to supress tumour growth. Since the tumour suppressor capacity of PTEN depends on its ability to inhibit the Akt pathway (Georgescu 2010; Cantley and Neel 1999; Furnari, Huang, and Cavenee 1998; Phadngam *et al*. 2016; Carnero and Paramio 2014) these results suggest that these mutants show reduced ability to inhibit Akt. Consistent with this, PTEN-WT expression in PC-3 cells resulted in ~67-69% suppression of Akt phosphorylation; however, cells infected with the K266R mutant did not have significantly different Akt phosphorylation levels compared to control cells lacking PTEN. Furthermore, the K254R mutant was able to supress Akt phosphorylation by only ~38-39% compared to control (Huang *et al*. 2012). Huang *et al*. (2012) also reported that compared to WT, PTEN K266R mutant showed dramatically less phosphate release in Malachite green phosphatase assays. Staining for PIP_3 in confocal imagining experiments in PC-3 stable cell lines expressing PTEN-WT or K266R revealed an increase of PIP_3 at the membrane in K266R-expressing cells. Therefore, the SUMOylation of PTEN plays an important or even critical role in

PTEN phosphatase activity with downstream effects against Akt and tumour suppressive activity, depending on the site of conjugation (Huang *et al*. 2012).

I therefore wanted to compare activity of WT and PTEN-3KR against the P-Akt pathway, to see how enhanced SUMOylation of PTEN would regulate P-Akt. Taking into account work by Huang *et al*. the K254R and K266R mutations in PTEN-3KR should block the ability of PTEN to inhibit the Akt pathway.

5.1.5 PTEN Dimerization and Effects of SUMOylation

PTEN can form homodimers *in vitro*; the dimer interface is formed by the two phosphatase domains (Heinrich *et al*. 2015). Dimerized PTEN is also more catalytically active than monomeric PTEN, and has a higher affinity for the membrane (Heinrich *et al*. 2015; Papa *et al*. 2014). As PTEN catalytic activity is critical to LTD, and PIP_3 can regulate AMPAR localisation, (Arendt *et al*. 2010; Jurado *et al*. 2010), a point of interest is how SUMOylation might influence dimerization.

Catalytically inactive PTEN mutants can hetero-dimerize with WT and have a dominant-negative effect in limiting phosphatase activity (Papa *et al*. 2014). It was reported that one mutated PTEN allele can have a stronger effect on tumour phenotypes and p-Akt regulation than deletion of one allele, so PTEN mutations can be “worse than nothing”, in the sense that having a mutated form of PTEN can be worse than having one missing PTEN allele (Papa *et al*. 2014; Leslie and den Hertog 2014). Papa *et al*. (2014) examined PTEN mutant G129E which retains protein phosphatase activity but lacks phosphoinositide phosphatase activity, and PTEN C124S mutant which lacks all phosphatase activity (Myers *et al*. 1998; Myers *et al*. 1997; Myers and Tonks 1997). Compared to PTEN-WT and $PTEN^{+/-}$ cells, PTEN $G129E^{/+}$ or PTEN $C124S^{/+}$ cells showed lower ability to regulate PI3K/Akt pathway. Mice with these mutations also showed higher tumour progression (Papa *et al*. 2014). It concluded that mutants can dimerize with WT shown in immunoprecipitation experiments, and suppress WT activity against PIP_3 shown in phosphatase assays where PIP_3 was used as a substrate (Papa *et al*. 2014). Considering this, I wanted to test the ability to PTEN-3KR to dimerize with PTEN-WT. It was reported that C-terminal tail phosphorylation negatively regulates PTEN dimerization but effects of SUMOylation on dimerization has not been tested. It is also suggested that C-terminal tail phosphorylation negatively regulates dimerization and causes dimer destabilization (Heinrich *et al*. 2015; Papa *et al*. 2014). Furthermore, polyubiquitination of PTEN at K342 and K344 has been shown to reduce dimerization as well as membrane localisation and ability to limit the Akt pathway (Lee *et al*. 2019).

These studies together show that phosphorylation and ubiquitination can regulate PTEN dimerization, and dimerization can regulate PTEN's localisation and catalytic activity against PIP_3 (Papa *et al*. 2014; Heinrich *et al*. 2015; Lee *et al*. 2019).

5.1.6 Effects of SUMOylation on PTEN Stability

Due to the finding that PTEN-3KR is more ubiquitinated and SUMOylated, I also wanted to examine the rate of its degradation. I expected it to be less stable than WT, as ubiquitination of PTEN is reported to reduce its stability (Leslie and Gupta, 2016). Wang *et al*. (2014) reported that SUMOylation at K254 and K266 enhances stability, by blocking ubiquitination and subsequent degradation, but the effects of PTEN SUMOylation on stability have not been widely tested.

Furthermore, as explained in Section 1.5.6, ubiquitination of PTEN may be involved in PTEN loss seen in AD brains (Kwak *et al*. 2010).

5.1.7 PTEN in Synaptic Plasticity- AMPAR Trafficking in Neurons

AMPAR trafficking is a process essential in plasticity, and that has been reported to be defective in AD (Chang *et al*. 2006; Shi *et al*. 2001; Passafaro, Piech, and Sheng 2001). As reviewed in the Introduction, the lipid phosphatase activity of PTEN is critical in NMDAR-dependent, hippocampal LTD; LTD is blocked by pharmacological PTEN inhibition or expression of a PTEN-G129E mutant that lacks lipid phosphatase activity, but retains has protein phosphatase activity (Myers *et al*. 1998; Jurado *et al*. 2010). PTEN localizes at the PSD after NMDAR activation, where it associates with PSD-95 (Jurado *et al*. 2010). Jurado *et al*. (2010) suggest this enables PTEN to be positioned near the membrane at activated synapses, which would improve its catalytic activity by allowing access to PIP_3, which is critical in AMPAR clustering at the membrane (Arendt *et al*. 2010). Furthermore, PTEN has been shown to directly downregulate the increase in PIP_3 levels after plasticity events (Arendt *et al*. 2014).

Conversely, overexpression of PTEN-WT can reduce AMPAR EPSCs under basal conditions, without affecting subsequent LTD induction (Jurado *et al*. 2010). Jurado *et al*. (2010) suggest this could because overexpressed PTEN is either acting on a different pool of AMPARs to those involved in LTD, or that the suppression of AMPARs by PTEN overexpression is not able to saturate LTD expression.

Liu *et al*. (2013) provide more evidence of PTEN's role in AMPAR trafficking in hippocampal neurons in a model of stretch injury, where neurons are grown on silicone which is then stretched, used to simulate conditions under traumatic brain injury (Geddes *et al*. 1995). PTEN protein and mRNA levels were increased after stretch injury, which was associated with reduced

GluA2 surface expression. Furthermore, PTEN inhibition with bisperoxovanadium was able to

enhance neuron survival by inhibiting the reduction of surface GluA2 (Liu *et al*. 2013), suggesting that enhanced PTEN levels are directly damaging in this model of traumatic brain injury.

PTEN's mechanism of action in neurotransmission and NMDAR- Dependent LTD is reliant on its phosphatase activity against PIP_3, and PIP_3 levels also regulate AMPAR trafficking by enabling AMPAR clustering at the membrane (Jurado *et al*. 2010; Arendt *et al*. 2010; Arendt *et al*. 2014). PTEN's phosphatase activity against PIP_3 is reported to be regulated by SUMOylation (Huang *et al*. 2012), so it's possible that SUMOylation of PTEN could have influence on NMDAR-Dependent LTD and AMPAR trafficking. Given the lack of phosphatase action against PIP_3 from PTEN-reduced SUMOylatable mutants (Huang *et al*. 2012), it will be interesting to test the effects of PTEN-3KR with enhanced SUMOylation in the context of AMPAR trafficking, which has not yet been assessed.

5.2 Aims

My data indicate that that mutation of 3 lysines in PTEN (K254R/K266R/K289R; PTEN-3KR) results in enhanced SUMOylation and ubiquitination of PTEN. I therefore reasoned that further examination of this mutant would be informative of the effects of SUMOylation on PTEN localization, stability, dimerization and catalytic activity. I aimed to characterize these parameters in cell lines, and compare WT and PTEN-3KR localization in neurons. I also wanted to test surface GluA2 expression under PTEN knockdown, and WT or PTEN-3KR rescue conditions, to further understand how PTEN might influence plasticity through AMPAR trafficking, and to determine whether PTEN SUMOylation and ubiquitination are involved. I also aimed to test whether changes to global SUMOylation in neurons could alter AMPAR expression.

Specifically, my aims were to

- Characterise the PTEN-3KR mutant as a tool to study PTEN SUMOylation and ubiquitination in terms of its stability, localisation and ability to dimerize in cell lines and/or neurons
- Create a stable cell line to characterize the phosphatase activity of PTEN-3KR
- Examine whether enhancement of global SUMOylation in neurons through SENP3 KD can influence AMPAR levels
- Examine the role of PTEN on AMPAR trafficking in neurons, and determine whether PTEN-3KR has different functional effects to WT

5.3 Results

5.3.1 PTEN-3KR and WT are Degraded at a Similar Rate in HEK293T Cells

Due to previous work showing the role of SUMO and ubiquitin in PTEN stability (Wang *et al*. 2014; Gupta and Leslie, 2016), and given that PTEN-3KR is both more SUMOylated and ubiquitinated than WT, a cycloheximide timepoint experiment was carried out to compare stability of these constructs. Cycloheximide is an inhibitor of protein synthesis; it binds the ribosome, preventing translocation (Obrig *et al*. 1971; Schneider-Poetsch *et al*. 2010). It can therefore be used to determine the half-life of a protein, and examine how quickly a protein is turned over after protein synthesis is inhibited (Kao *et al*. 2015).

HEK293T cells were transfected with GFP-tagged PTEN-WT or PTEN-3KR, and the next day treated with cycloheximide. 24 hours later they were lysed, subject to Western blot and blotted for GFP. There was no significant difference in the total amount of PTEN-WT or PTEN-3KR remaining after 24h, suggesting they are both being degraded to a similar extent. This is in contrast to findings that ubiquitinated PTEN is less stable (Gupta and Leslie, 2016). However, Gupta and Leslie (2016) suggest K66 (which is not mutated in PTEN-3KR) is the most significant site in terms of stability, and it is not clear which sites are more ubiquitinated in PTEN-3KR.

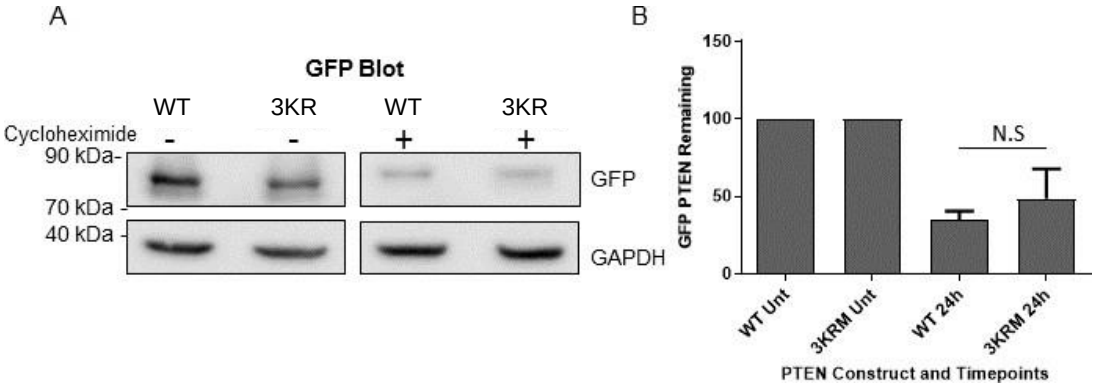

Figure 5.3.1.1. WT and PTEN-3KR show similar levels of turnover in 24 hours.

A) HEK 293T cells were split into a 12 well dish. The next day, cells were transfected with 1µg of either GFP-tagged PTEN-WT or PTEN-3KR DNA. 24 hours later 100µg/mL cycloheximide was added. Cells were incubated for another 24 hours then lysed in 1x SDS loading buffer and subject to Western blot. B) Quantification of A: cycloheximide-treated samples were normalised to untreated samples as a control, graph shows percentage of remaining signal compared to untreated samples. Samples were normalised to GAPDH as a loading control. PTEN-WT and PTEN-3KR were compared using an unpaired t-test with Mann Witney correction. There was no significant difference between total levels of PTEN-WT and PTEN-3KR expressing cells after 24h cycloheximide treatment (p=0.208, N=4).

5.3.2 PTEN-3KR and WT do not Dimerize Differently with PTEN-WT in HEK293T Cells

Due to evidence that PTEN mutants can hetero-dimerize with PTEN-WT and obscure its ability to regulate the Akt pathway (Papa *et al*. 2014), I wanted to test whether PTEN-3KR would dimerize differently with WT. To do this, Tandem Affinity Purification (TAP) tagged WT-PTEN was expressed with either GFP, GFP-tagged WT-PTEN or 3KR-PTEN, and a GFP trap was then carried out and the samples blotted for the SBP-tag in TAP-PTEN. The TAP tag contains Streptavidin-Binding Peptide (SBP) and Calmodulin-Binding Peptide (CBP) tags. GFP-tagged WT-PTEN and 3KR-PTEN immunoprecipitated similar amounts of WT-TAP-PTEN. This suggests PTEN-3KR can also dimerise with WT, and there was no significant difference in dimerization between WT/WT and WT/PTEN-3KR (P=0.963, N=3) (Figure 5.3.2.1).

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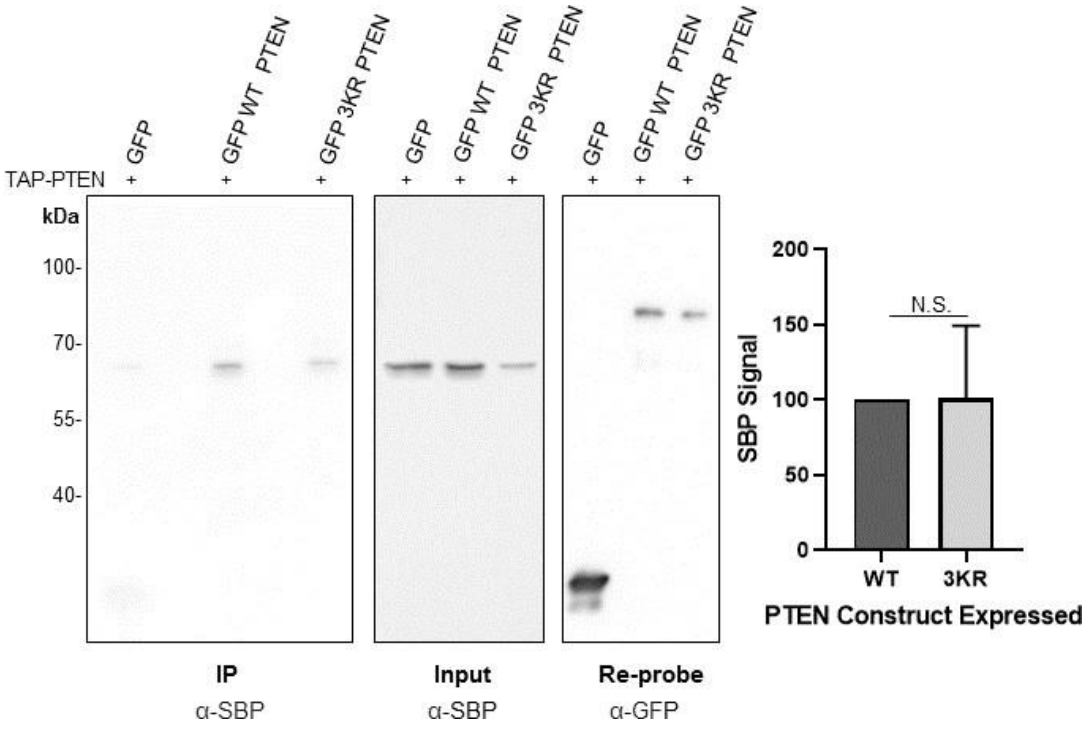


Figure 5.3.2.1. WT and PTEN-3KR Dimerize with PTEN-WT to a Similar Extent.
6cm dishes of HEK cells were transfected with TAP-WT-PTEN along with GFP, GFP-tagged WT-PTEN or 3KR-PTEN (TAP PTEN is both SBP and CBP tagged). Two days later, cells were lysed and subject to immunoprecipitation via GFP-trap and blotted for SBP and GFP. SBP was normalised to GFP, then PTEN-3KR binding with WT was expressed as a percentage of WT binding with WT. A One-sampled t-test showed there was no significance difference between conditions (P=0.963, N=3).

5.3.3 PTEN-3KR has Reduced Ability to Limit the Akt Pathway in HeLa Cells

PTEN mutants with reduced SUMOylation capacity have been reported to have limited ability to supress Akt activation (Huang *et al*. 2012). To compare p-Akt suppression between WT and PTEN-3KR conditions, I created HeLa cell lines Lentivirally expressing GFP-tagged plasmids with either Scrambled shRNA (Scr), PTEN knockdown shRNA (KD), or knockdown-rescue GFP-tagged PTEN-WT or PTEN-3KR (Figure 5.3.3.1.A). To create this cell lines, 40,000 HeLa cells were plated into wells of a 12 well plate. Four hours later 250µl Scr, PTEN KD, GFP-tagged PTEN-WT, or PTEN-3KR Lentivirus was added to separate wells. Three days later, cells were split into T75 flasks and allowed to grow until confluent. Blotting of these cells for PTEN showed clear expression of GFP-tagged WT and PTEN-3KR and confirmed knockdown of endogenous PTEN, and overexpression of the GFP-PTEN replacements (Figure 5.3.3.1.A).

Akt and Phosphorylated (active) Akt were then blotted for in these cell lines (Figure 5.3.3.1.C.). Consistent with previous reports (Cantley and Neel 1999; Manning and Cantley 2007), PTEN KD cells show enhanced Akt activation, evidenced by the large increase in p-Akt (Ser473) compared to Scr control. Furthermore, cells expressing PTEN-3KR also showed enhanced p-Akt levels normalised to total Akt, compared to cells expressing WT-PTEN. This suggests in PTEN-3KR conditions there is a higher p-Akt/ total Akt ratio, which is commonly used to measure Akt activation (Griffin *et al*. 2005). PTEN-3KR is therefore less able to supress Akt activation. However, p-Akt levels were reduced by PTEN-3KR expression compared to cells lacking PTEN, suggesting this mutant is not completely inactive. This is in line with Huang *et al*. (2012) who also showed single mutations at SUMO sites K254R and K266R (two of the three mutations also in PTEN-3KR) reduces its activity against Akt. However, I note that the group state that this deficiency is due to reduced SUMOylation of PTEN, while my results show PTEN-3KR exhibits enhanced SUMOylation (Figure 4.3.4.1).

Interestingly, total Akt was significantly increased in WT-PTEN expressing cells compared to control (Scr) cells, but reduced in PTEN-3KR-expressing cells. Potentially, these results suggest the cell could be trying to compensate for the enhanced inhibition of p-Akt resulting from WT-PTEN overexpression by producing more Akt. This could be tested by expressing a catalytically dead form of PTEN and examining whether the same effect is seen. Conversely, cells expressing the PTEN-3KR mutant exhibit enhanced Akt activity, and may thus respond by reducing total Akt levels.

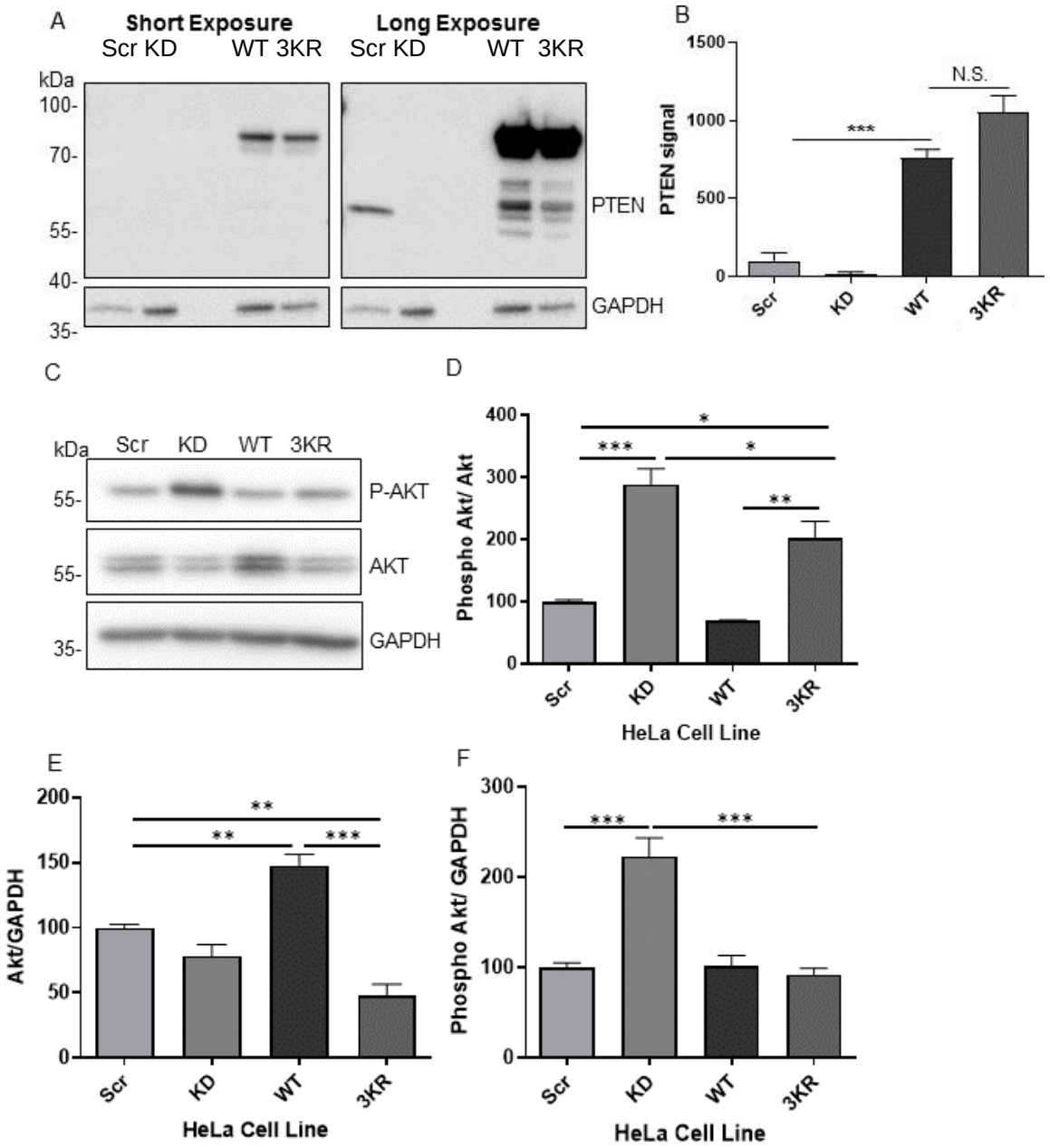


Figure 5.3.3.1. HeLa cell lines expressing PTEN-3KR have Significantly Increased P-Akt/ Akt Ratio Compared to PTEN-WT Cells.

A) Representative blot of HeLa stable cell lines. 300,000 cells were plated into each well of a 6 well dish. When confluent, cells were then lysed in sample buffer, subject to Western blot and blotted for PTEN. B) Quantification of A. PTEN was normalised to GAPDH. Each experiment was normalised to the mean of each experiment, then one-way ANOVA with Tukey correction was used to assess significance (N=3). C) Representative blot showing Akt, phosphorylated Akt (site S473) in samples of the cell lines shown in A. D/E) Quantification of Akt, P-Akt and P-Akt/Akt. Samples were run on gels separately, one membrane of each was blotted for P-Akt and the other was blotted for Akt and GAPDH. P-Akt was normalised to Akt for quantification of P-Akt. Akt was normalised to GAPDH for quantification of Akt. Each experiment was normalised to the mean of each experiment, then One-way ANOVA with Tukey correction was then carried out to assess significance (N=3, *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$).

5.3.4 Phospho-Erk is not Significantly Altered in PTEN-3KR Versus PTEN-WT Expressing HeLa Cells

Extracellular Signal-Regulated Kinase (Erk) is part of a signal transduction pathway which mediates various cellular processes including cell cycle progression and cell proliferation (Yoon and Seger 2006). Erk is also involved in plasticity through regulating GluA2 delivery to synapses (Qin *et al*. 2005). There is evidence that PTEN can negatively regulate Erk phosphorylation (Weng *et al*. 2002), so I wanted to see if phospho-Erk (p-Erk) level would change between conditions in my cell lines. Samples of Scr, PTEN KD, PTEN-WT and PTEN-3KR were blotted for p-Erk and Erk. Although both an Erk and p-Erk antibody were used, only the phospho-Erk was successful in detecting bands at the right molecular weight. This is a double band at 44kDa and 42kDa, which are p-Erk1 (T202/T204) and p-ERK2 (T185/T187) respectively (Sigma datasheet Product Number E7028). For this reason, p-Erk has been normalised to GAPDH. There is no significant difference between any of the conditions, suggesting PTEN levels are not influencing p-Erk levels in these cell lines (Figure 5.3.4.).

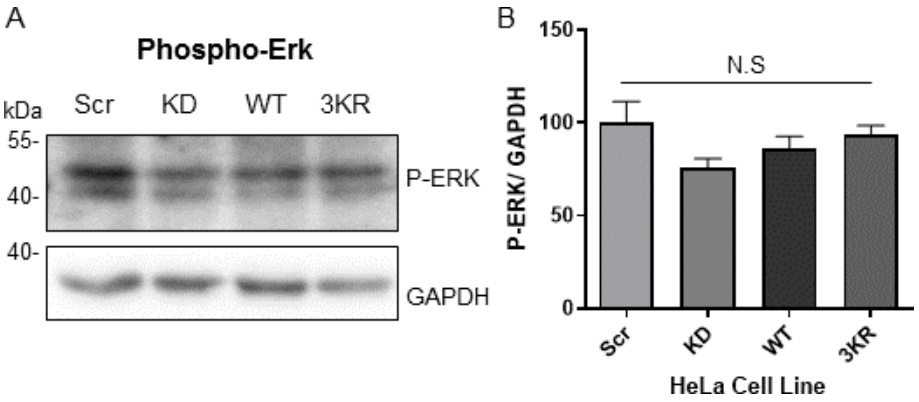
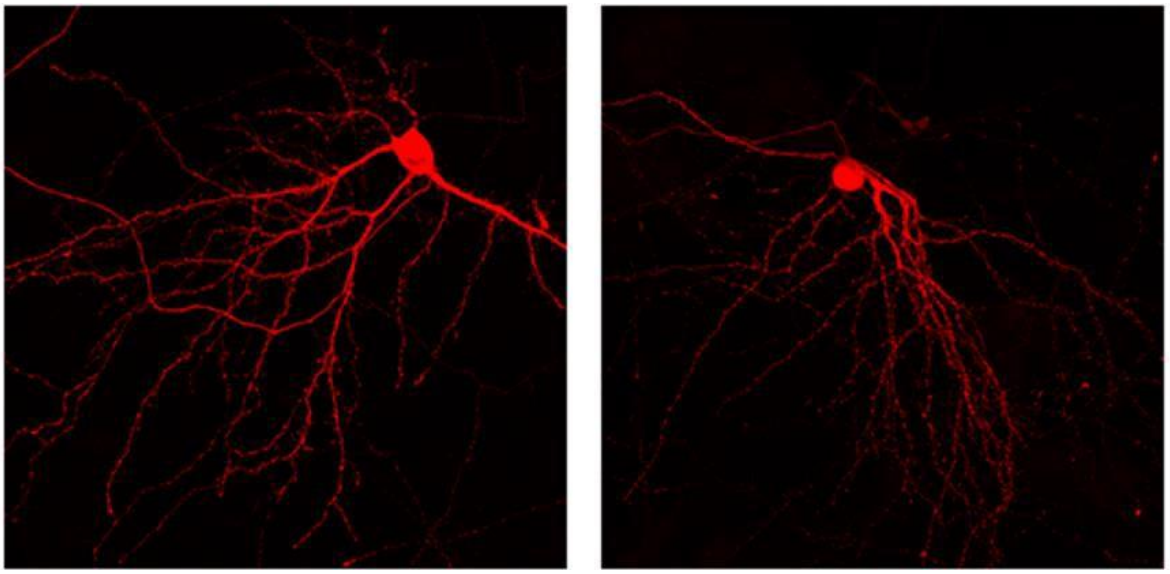


Figure 5.3.4.1. Phospho-Erk activity is unaffected by manipulation of PTEN.

Samples from Scr, PTEN KD, PTEN-WT and PTEN-3KR HeLa cell lines shown in Figure 5.3.3. were blotted for phospho- Erk (p-ERK1 (T202/T204) and p-ERK2 (T185/T187) and GAPDH. P-Erk was normalised to GAPDH. All experiments were individually normalised to the mean of each experiment, then a one-way ANOVA with Tukey correction was used to analyse significance (N=3).

5.3.5 Imaging of PTEN-WT and PTEN-3KR in Neurons

There is evidence that PTEN with K254R and K266R mutations at SUMOylation sites localise to different parts of the cell in cell lines compared to WT (Huang *et al*. 2012). I therefore compared the localisation of GFP-tagged WT and PTEN-3KR constructs in neurons, which has not previously been examined. Transfection of constructs into hippocampal neurons and subsequent fixing and imaging showed that both proteins show an extremely diffuse expression pattern, making it difficult to establish any differences in localisation. Nonetheless there were no obvious differences in localisation between conditions, however it is noted that to improve validity, neurons with a lower expression of the constructs should be imaged. However, I decided to instead focus on biochemistry experiments, as these provided more information on the function of PTEN-3KR.



PTEN-WT PTEN-3KR

Figure 5.3.5.1. Imaging of WT and PTEN-3KR in Hippocampal Neurons.
Hippocampal neurons were transfected on DIV 8/9 with GFP-WT-PTEN or GFP-3KR-PTEN. 4/5 days later, neurons were fixed and stained with GFP antibody before confocal imaging. Image shows representative neurons from 2 independent cultures.

5.3.6 SENP3 Knockdown does not Influence Total AMPAR Levels in Neurons

PTEN has previously been shown to play a role in AMPAR trafficking (Liu *et al*., 2013); as a starting point to begin examining the role of PTEN SUMOylation in this, I tested whether SENP3 KD could alter AMPAR levels. KD of SENP can be used to model enhanced SUMOylation conditions (Bawa-Khalfe *et al*. 2010). SENP3 can deconjugate SUMO2/3, and to a lesser extent, SUMO1, so it was expected that SENP3 KD would enhance global SUMOylation by blocking de-SUMOylation (Gong and Yeh 2006; Yeh 2009). SENP3 KD Lentivirus, made by Dr K Wilkinson, was added to neurons and left for 7 days before lysis and blotting. SENP3 levels had no significant effect on GluA1 or GluA2 total levels. This suggests that level of global SUMOylation in cortical neurons is not influencing total AMPAR expression.

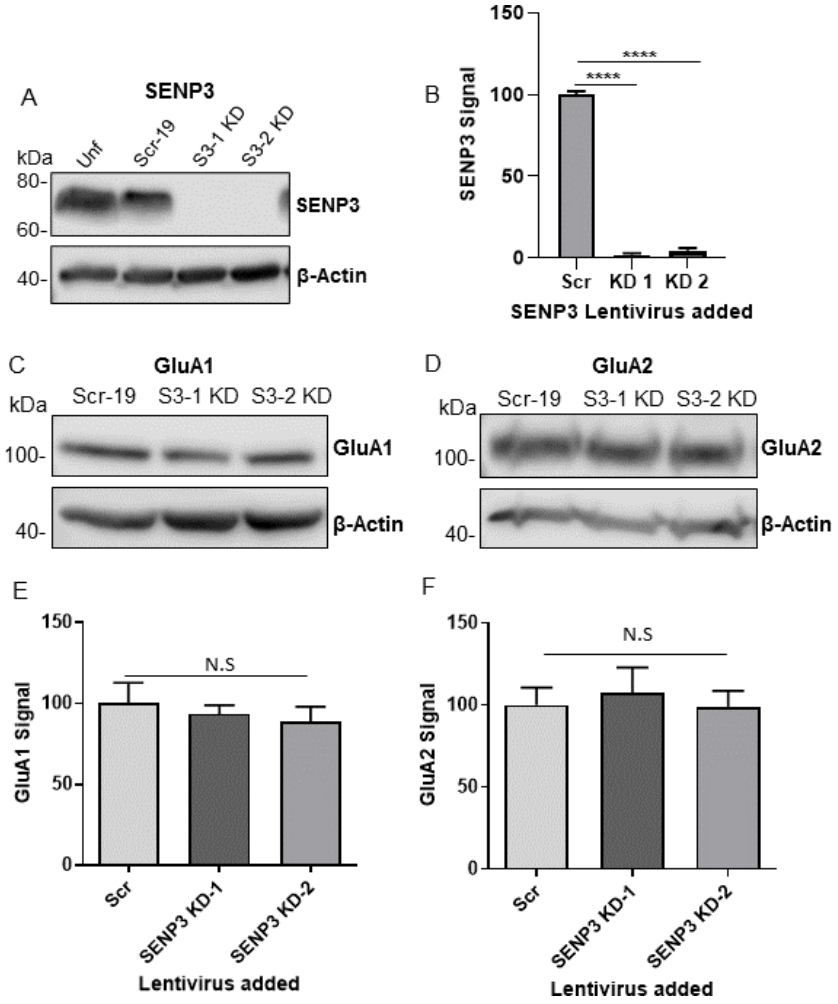


Figure 5.3.6.1. SENP3 Knockdown does not Significantly Alter GluA1 or 2 Levels in Neurons.

(A) Representative blot of SENP3 expression after SENP3 Lentiviral KD by two shRNA KD viruses: SENP3 KD-1 (KD-1) or SENP3 KD-2 (KD-2) in neurons. Neurons were infected on DIV8-12, 7 days later were lysed and subject to Western blot. B) Quantification of A: samples were individually normalised to the mean of each experiment, then analysed in a one-way ANOVA with Dunnett's correction (both KDs were compared to Scr as a control). C/D) Samples show in A) were blotted for GluA1 and GluA2. E/F) Quantification of C) and D). GluA2 was normalised to β-Actin or GAPDH, then each experiment was normalised to the mean of that experiment, then analysed using one-way ANOVA with Dunnett's correction comparing KD samples to Scr (CI= 95%) (N=5; *=p ≤ 0.05, **=p ≤ 0.01, ***=p ≤ 0.001, ****=p ≤ 0.0001).

5.3.7 Lentiviral PTEN KD Significantly Reduces Surface GluA2 Levels in DIV 20-22 Neurons

To further investigate role for PTEN in AMPAR trafficking, surface biotinylation was conducted to establish whether PTEN KD could influence surface or total AMPAR levels. PTEN has previously been reported to regulate AMPAR surface levels (Liu *et al*. 2013), Furthermore, PIP_3 levels are critical in AMPAR localisation at the membrane (Arendt *et al*. 2010), so as PTEN-3KR had reduced phosphatase activity against p-Akt, I reasoned this could regulate AMPAR levels at the membrane differently to WT.

After 7-day lentiviral PTEN knockdown, or treatment with Scr control, DIV 20-22 cortical rat neurons were surface biotinylated, and labelled surface proteins isolated using streptavidin-beads before Western blotting of surface and total samples (Figure 5.3.7.1.). Lentiviral KD of PTEN significantly reduced the proportion of GluA2 expressed on the surface, without significantly affecting total GluA2 levels.

This is in contrast to Lui *et al*. (2013) who reported that increases in PTEN are associated with a loss of GluA2 at the surface. PTEN inhibition was able to limit the reduction of GluA2 lost from the surface after stretch injury, so overall in this context, PTEN negatively regulated surface GluA2 (Liu *et al*. 2013). However, PTEN KD was not tested in that study, instead BpV was used to transiently block PTEN activity. Moreover, there were other methodological differences, my experiments involved surface biotinylation of cortical neurons to measure surface GluA2, while Liu *et al*. (2013) conducted imaging on hippocampal neurons.

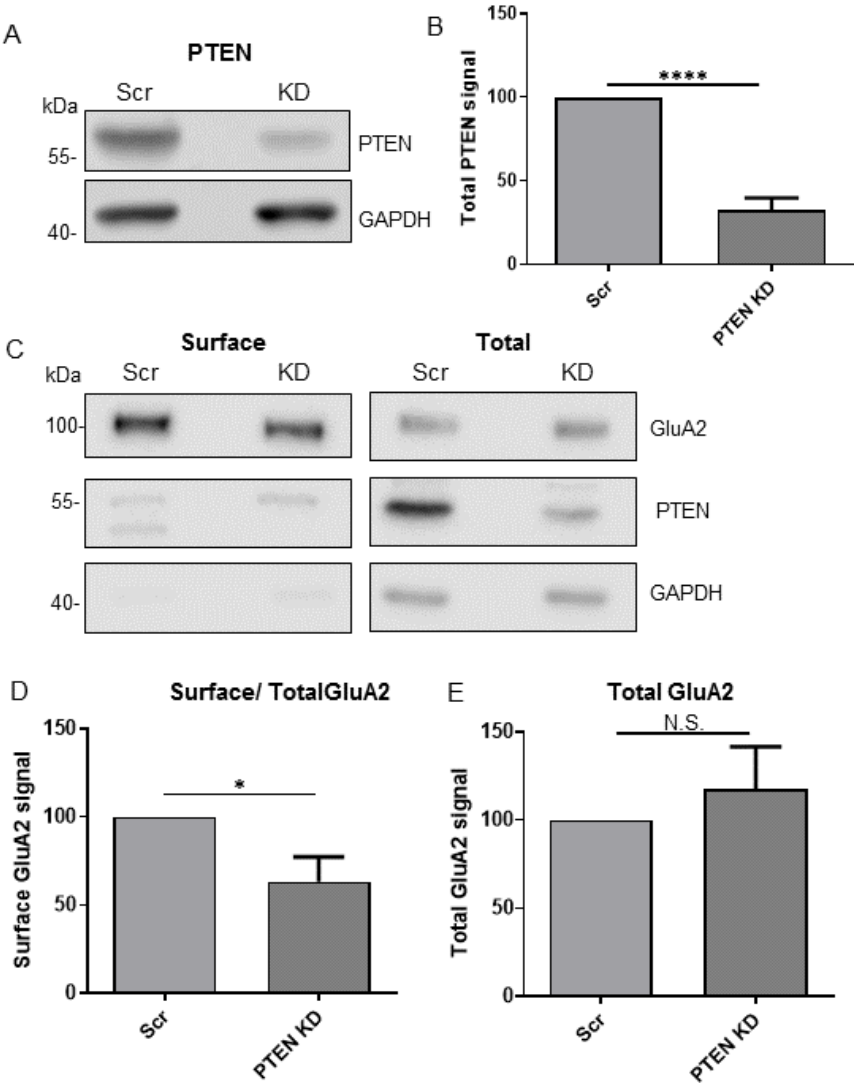


Figure 5.3.7.1. Lentiviral PTEN KD Significantly Reduces Surface GluA2 Levels in DIV 20-22 Neurons.

(A) Representative blot showing PTEN KD after 6-7 day lentiviral infection. Cortical neurons were infected on DIV 14-16 with Scr or KD lentivirus. (B) Quantification of blot on the left, showing PTEN total signal after PTEN KD compared with Scr ($p<0.0001$, N=7). (C) Representative blot of surface biotinylation after PTEN KD. Scr and PTEN KD lentiviruses were added to DIV 14-16 cortical neurons for 6-7 days, then surface biotinylation was carried out. (D) Quantification of GluA2 total signal after PTEN KD ($p=0.484$, N=7). (E) Quantification of GluA2 surface blot above. Surface samples were normalised to total samples and Scr was compared to KD ($p=0.0397$, N=7). In all analyses, Scr and KD were compared using a One sampled t-test where Scr was set to a hypothetical value of 100. Surface was normalised to total and GluA2 totals were normalised to GAPDH as a loading control.

5.3.8 PTEN-WT and PTEN-3KR do not Bind Myc-PSD-95 Significantly Differently in HEK293T Cells

PSD-95 is important for clustering of AMPARs at synapses (Chen *et al*. 2000; Bats, Groc, and Choquet 2007). Furthermore, PTEN binding to PSD-95 is reported to be critical in NMDAR-Dependent mediated LTD (Jurado *et al*. 2010). I therefore wanted to see if PTEN-3KR would bind PSD-95 differently to WT, to see if enhanced SUMOylation could influence this interaction. I expressed GFP-WT and PTEN-3KR with Myc-PSD-95 in HEK293T cells, then carried out immunoprecipitation and blotted for Myc and GFP. There was no statistical difference between WT and PTEN-3KR binding to Myc-PSD-95, suggesting enhanced SUMOylation on PTEN-3KR is not influencing binding to PSD-95.

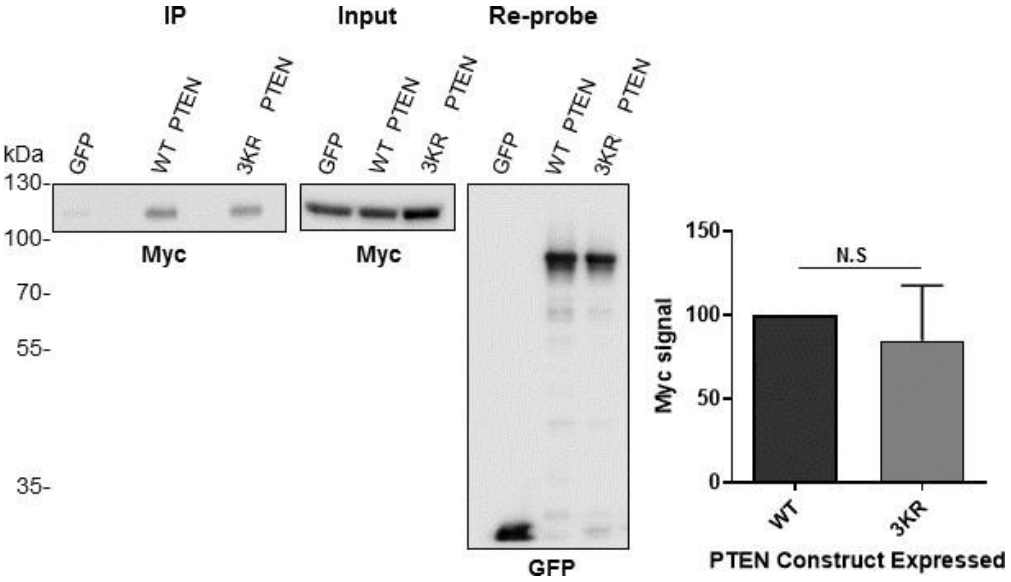


Figure 5.3.8.1. WT and PTEN-3KR do not bind Myc-PSD-95 significantly differently.

Representative blot showing immunoprecipitated Myc-PSD-95 in HEK cells. Cells were transfected with Myc-tagged PSD-95 along with GFP, PTEN-WT or PTEN-3KR and subject to immunoprecipitation and Western blot protocols (see Methods 3.4.1). Samples were blotted for Myc and GFP. Immunoprecipitated Myc signal was normalised to total Myc signal for each condition, then normalised to GFP to allow for differences in total expression levels of both proteins to be controlled for. PTEN-3KR was then compared with WT which was set to a hypothetical value of 100. A one sampled T-test was then used to establish significance ($p=0.690$, $N=3$).

5.3.9 Surface GluA2 is Unaffected by PTEN Knock Down in DIV 15-16 Neurons

To further explore effects of PTEN in AMPAR trafficking, the experiment was repeated with two more conditions: PTEN-WT-KD rescue and PTEN-3KR-KD rescue (both cloned by Dr K Wilkinson) (Figure 5.3.9.1). The purpose of the experiment was to see if expressing recombinant PTEN-WT after the KD could rescue effects of KD, which would validate the previous finding that PTEN KD influences GluA2 surface levels. The addition of the PTEN-3KR mutant-KD rescue also allowed me to examine whether enhanced SUMO or ubiquitin modification of PTEN could be involved in its effects on GluA2 expression. Given that PTEN-3KR could not regulate the P-Akt pathway (Figure 5.3.3.1), and given the involvement of PIP_3 levels in AMPAR anchoring at the synapse (Arendt *et al*. 2010), I postulated that PTEN-3KR may affect GluA2 differently than WT. The result was that the KD effect of reducing surface GluA2 did not replicate; there was no significant effect of any of the conditions when compared against each other. However, it is noted that the neurons were younger in this experiment than the previous experiment (infected on DIV 8-10 rather than DIV 14-16). As gene transfer of viral constructs is more effective in younger neurons (Levin, Diekmann, and Fischer 2016), I used these younger neurons in this experiment to improve the level of rescue expression. However, this may have confounded the results, given that past DIV 14, neurons are considered to be at the "*late mature"* stage, where the neuronal network is stable and synaptic transmission is in its prime (Moutaux *et al*. 2018). Considering this with the importance of PTEN in synaptic development (Fraser *et al*. 2008; Kwon *et al*. 2006), reviewed in 1.2.13), this may explain the variation between this experiment (Figure 5.3.8.1.) and the previous (5.3.7.1.).

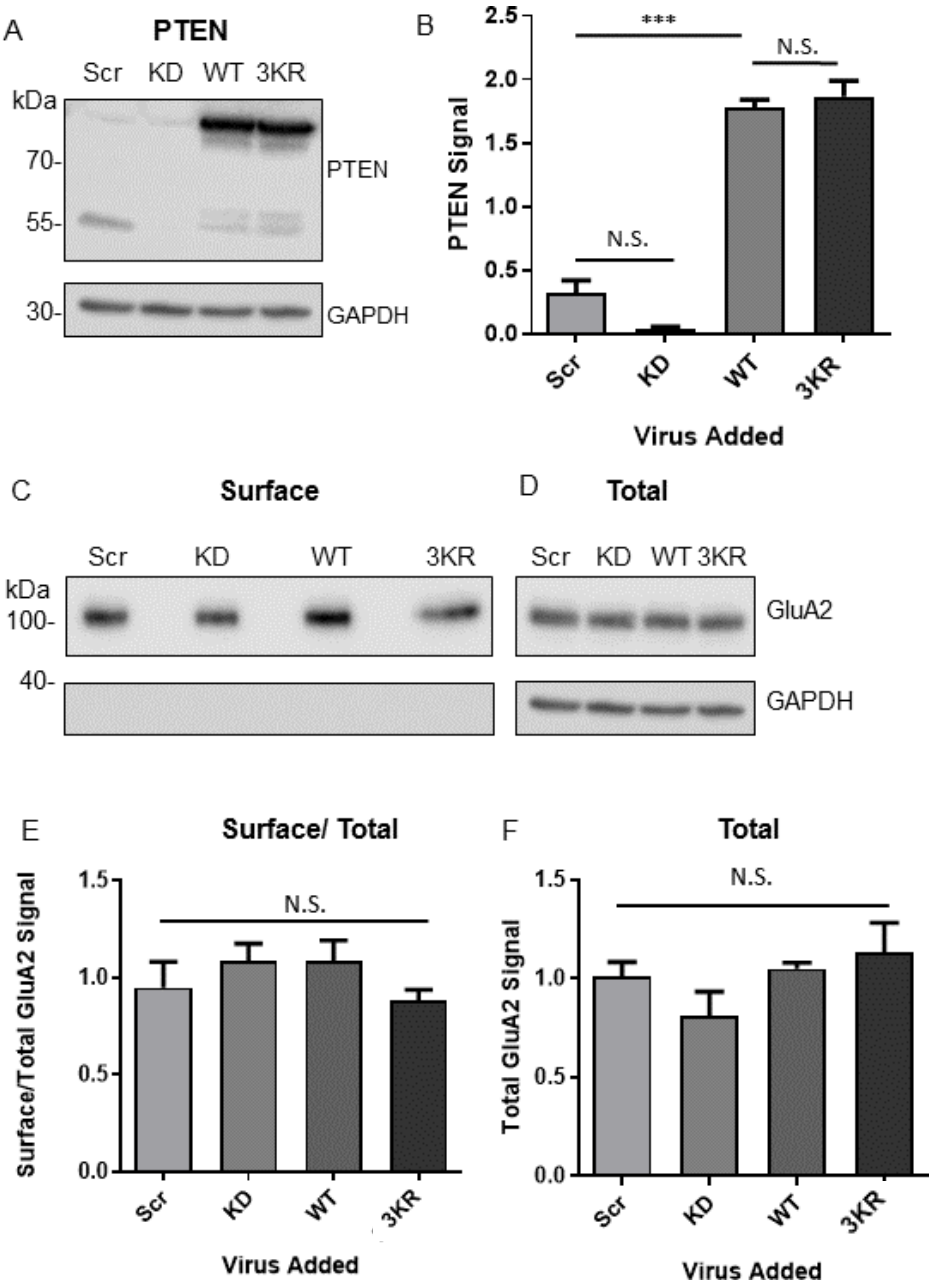


Figure 5.3.9.1. PTEN KD, or expression of WT or PTEN-3KR, does not alter surface or total GluA2 levels in DIV 15/16 Neurons.

(A) Representative blot of PTEN level after surface biotinylation of DIV 15/16 cortical neurons after 6/7 day viral infection with GFP-tagged Scr, PTEN KD, PTEN-WT and PTEN-3KR Lentiviruses. B) Quantification of PTEN level in each condition, normalised to GAPDH. All experiments were individually normalised to the mean of each experiment, then all conditions were compared against each other using a One-way ANOVA with Tukey correction. PTEN levels differed significantly between Scr and WT or PTEN-3KR (****=p ≤ 0.0001, N=4). There was no significant difference in PTEN level between WT and PTEN-3KR or Scr and KD. C and D) Representative blot of surface and total GluA2 levels after surface biotinylation. E and F) Quantification of GluA2 total (N=4) and surface (N=5) signal after surface biotinylation. Surface GluA2 samples were normalised to total GluA2 samples, total GluA2 was normalised to GAPDH. All experiments were individually normalised, then all conditions were compared against each other using a One-way ANOVA with Tukey correction. No significant differences were found in GluA2 surface or total expression between conditions.

5.3.10 WT and PTEN-3KR Expression Increases Surface GluA2 in DIV20-22 Neurons

As the effect of the knockdown did not replicate in younger neurons, I decided to repeat these experiments in older neurons, to match the experiments shown in Figure 5.3.7.1. This meant that at the time the virus was added (DIV 14/15), neurons should already be mature (Moutaux *et al*. 2018). Unexpectedly, once again the decrease in surface GluA2 in PTEN KD seen in Figure 5.3.7.1 was not observed. However, both WT and PTEN-3KR overexpression significantly increased surface GluA2 levels. Taken together, these experiments suggest that PTEN level is having an effect on GluA2 surface expression, but the effect is not robust. The variation in results could be due to differences arising from different cell culture reagents such as horse serum or B27 in neuronal cultures having an effect on growth/health of neurons, or differences in density or activity of neurons. There is evidence that different dissections of cortical cultures can vary considerably in neuronal activity patterns, due to either differences in the rat which the cultures came from, or from later in development (Wagenaar, Pine, and Potter 2006).

There was no significant difference in surface GluA2 levels between WT and PTEN-3KR, suggesting enhanced SUMOylation/ubiquitination is not relevant to PTEN's ability to influence surface AMPAR expression. This is surprising given the defects in PTEN-3KR activity (Figure 5.3.3.1), and evidence that PIP_3 reduction limits postsynaptic AMPAR expression (Arendt *et al*. 2010). However, PTEN-3KR is not totally defective in its ability to supress p-Akt, so this lower level of activity could be enough to regulate AMPAR surface levels. It is also possible that the vast overexpression of PTEN-3KR and PTEN-WT from the KD-rescue constructs negates this difference; this overexpression would likely increase phosphatase activity and could cancel out potential differences between WT and PTEN-3KR at endogenous levels. Future experiments could include repeating this experiment with a lower level of expression to test this.

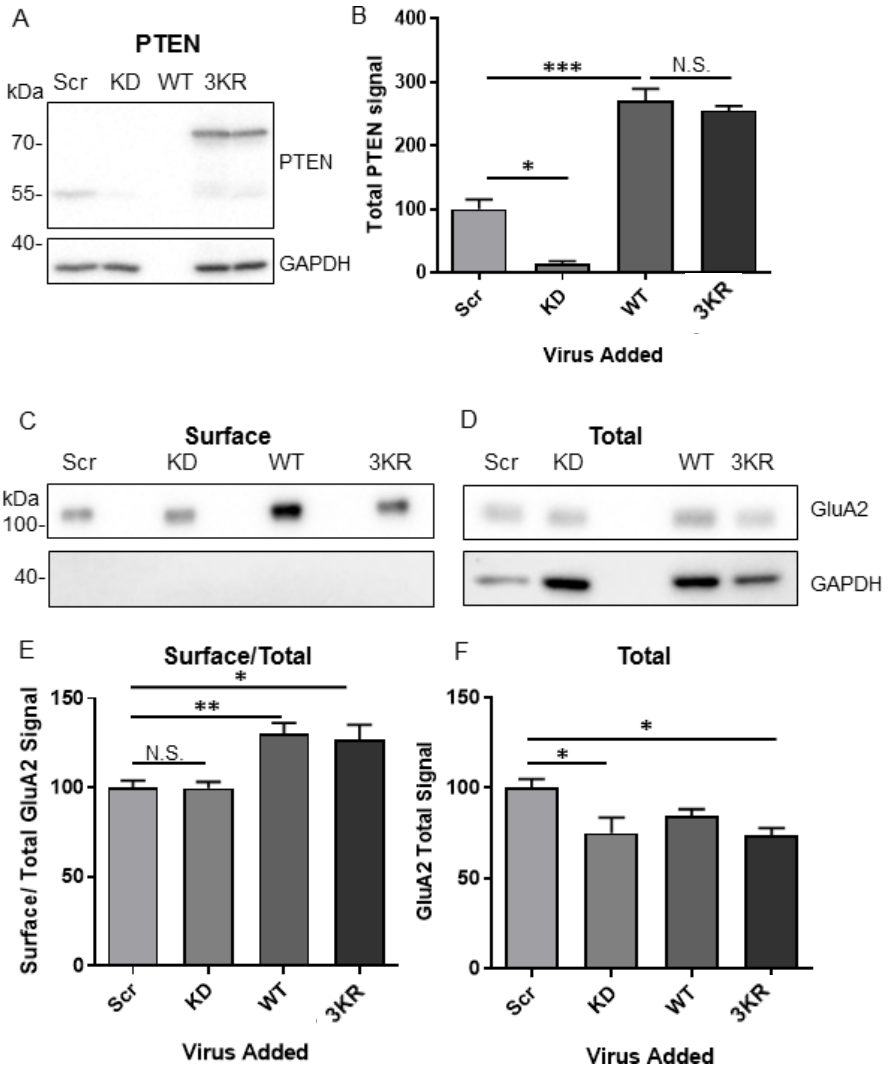


Figure 5.3.10.1. Lentiviral PTEN-WT and PTEN-3KR Overexpression Significantly Increase Surface GluA2 Levels in DIV 20-22 Neurons.

Cortical neurons were infected on DIV 14/15 and surface biotinylation protocol was carried out 6/7 days later. Samples were subject to Western blot protocols and blotted for GluA2 and GAPDH. A) Representative blot of PTEN expression after surface biotinylation of cortical neurons after 6/7-day viral infection with GFP-tagged Scr, PTEN KD, PTEN-WT and PTEN-3KR Lentiviruses. B) PTEN signal was normalised to GAPDH. Experiments were individually normalised to the mean of each experiment and analysed using One-way ANOVA with Tukey correction. C) and D) Representative blot showing surface and total GluA2 respectively. E) and F) Analysis of surface and total GluA2. Surface GluA2 was normalised to total GluA2 and total GluA2 was normalised to GAPDH. All experiments were individually normalised to the mean of each experiment. One-way ANOVA analysis was used with Tukey correction to analyse significance across all conditions (N=5, *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$, ****=$p \leq 0.0001$).

5.4 Discussion

5.4.1 PTEN Dimerization and Stability

In an attempt to reveal the effects of enhanced SUMOylation and/or ubiquitination on PTEN function, in this chapter I sought to characterise the functions and behaviour of PTEN-3KR, in terms of its regulation and effects on AMPAR trafficking.

I first compared PTEN-WT and PTEN-3KR in terms of stability and dimerization. Given that dimeric PTEN is more active than monomeric PTEN (Papa *et al*. 2014), and reduced-SUMO mutants are less catalytically active (Huang *et al*. 2012), I tested whether PTEN-3KR could dimerize with WT. No obvious difference was seen between conditions, suggesting WT and PTEN-3KR dimerize with WT to a similar extent.

Overexpression of SUMO ligase PIASxα reduces P-Akt in a PTEN-Dependent manner; this is suggested to be because PIASxα- driven SUMOylation increases PTEN stability (Wang *et al*. 2014). SUMOylation at K254 and K266 is thought to block ubiquitination and subsequent degradation (Wang *et al*. 2014). Other work has confirmed the role of ubiquitination in reducing stability and reported K266 to be the dominant site for stability (Wang *et al*. 2016; Leslie and Gupta, 2016). Therefore, PTEN-3KR could have been predicted to be more or less stable given that it is both more SUMOylation and ubiquitinated than PTEN-WT, and it is not known which sites SUMO and ubiquitin are conjugating to. No significant difference was found between degradation rate of WT and PTEN-3KR after cycloheximide treatment (Figure 5.3.1.1.), suggesting enhanced SUMO or ubiquitin modification in PTEN-3KR is not relevant to stability. This could be because the increased ubiquitin on PTEN-3KR is a Lys63 linked chain, rather than degradation-associated Lys48 (Kawadler and Yang, 2006; Haglund *et al.* 2003; Komander, 2009; Hicke *et al.* 1996). This could be tested by blotting with chain-specific ubiquitin antibodies.

5.4.2 PTEN-3KR, Localisation and the P-Akt Pathway

Ability to supress P-Akt is a well-documented ability of PTEN (Cantley and Neel, 1999; Manning and Cantley, 2007). Previous work has shown that PTEN mutants with reported reduced SUMOylation capacity show deficits compared to PTEN-WT in supressing P-Akt (Huang *et al*. 2012). It is therefore in line with previous work that PTEN-3KR could not regulate P-Akt as WT can (Figure 5.3.3.). However, previous work argues separate/ double mutations of PTEN at K266/K254/K289 reduce SUMOylation, whereas my results in chapter 3 show SUMOylation is increased.

Gonzales- Santamaria (2012) argue that SUMO forces PTEN into the “open” conformation by obscuring interactions between the C2 domain and C-tail, facilitating membrane localisation,

which is suggested to enable P-Akt regulation (Huang *et al*. 2012). This is based on the observation that viral infection causes translocation of PTEN to the membrane where it is SUMOylated by SUMO1 and 2. As mentioned, this paper tested PTEN-mutant SUMOylation level with tagged, recombinant SUMO, and results contrast with my own with endogenous SUMO. Therefore, my results suggest that this model may not be correct, and SUMOylation may inhibit membrane localisation, evidenced by lack of P-Akt regulation in PTEN-3KR. It was hoped that the imaging experiment (Figure 5.3.5.) could elucidate any differences between WT and PTEN-3KR localisation, but the expression levels were too high for any potential differences to be observed. Nonetheless, it seems mutation of the three known SUMO sites reduce PTEN's ability to regulate P-Akt. However, I argue that this is due to an excess of endogenous SUMO (or ubiquitin) on these mutants, rather than a lack of SUMO. This is in line with Shenoy *et al*. (2012), who argue SUMO would be more likely to hinder membrane localisation (This will be discussed further in the general discussion). Alternatively, it could be that PTEN SUMOylation must be tightly regulated, and extremes at either end of the scale can obstruct its activity. In addition to SUMO, it could be that effects seen are due to the increased polyubiquitination on PTEN-3KR, although my results do not support a difference in turnover rate of the PTEN-3KR mutant (Figure 5.3.1.).

Importantly, however, differences between PTEN-WT and PTEN-3KR cannot be unequivocally attributed to an increase of SUMO or ubiquitin on PTEN-3KR. It could be that mutations in PTEN-3KR have effects through another mechanism, such as a change in conformation (although molecular dynamic simulations by Huang *et al*. (2012) has shown K266R mutation does not induce conformational change). Repeating experiments in this chapter with the addition of SENP or a DUB would elucidate whether effects seen are due to enhanced SUMO/ubiquitin or another aspect of regulation.

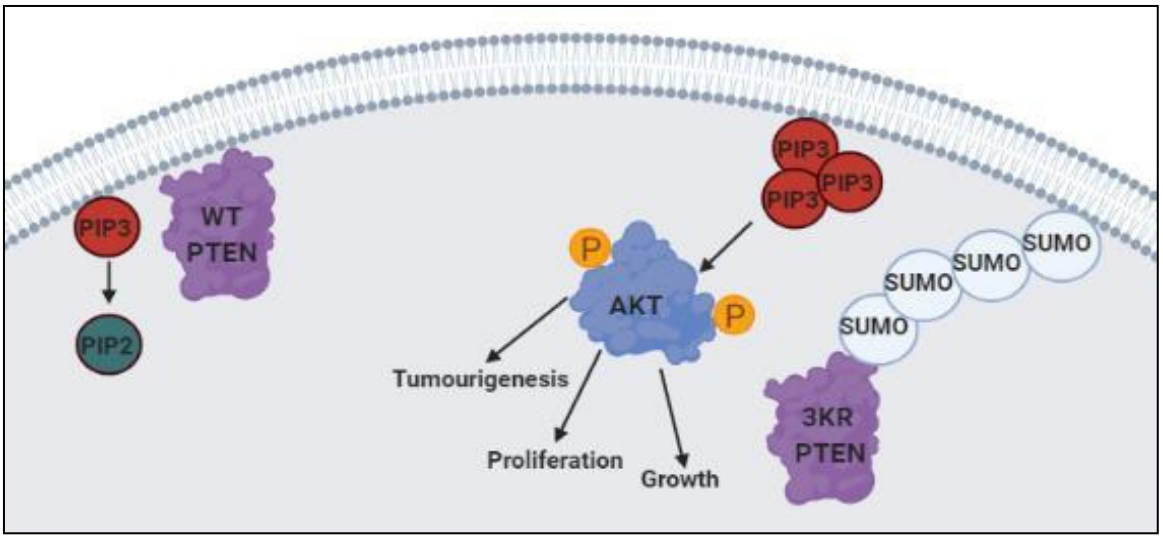


Figure 5.4.2.1. Schematic Representing the Possible Role of SUMO in Regulating PTEN Activity.

PTEN-WT at the membrane with phosphatase activity is capable of dephosphorylating PIP_3, reducing Akt activity (Maehama and Dixon 1998; Stambolic *et al*. 1998; Myers *et al*. 1998; Leslie *et al*. 2008). PTEN-3KR exhibits enhanced SUMOylation, and thus may be less able to be recruited to the membrane, and therefore unable to supress Akt activation, as membrane localisation is important in PIP_3 regulation (Leslie *et al*. 2008). Lack of suppression of P-Akt can lead to enhanced cell proliferation, growth and tumorigenesis (Stambolic *et al*. 1998; Cantley and Neel 1999; Shi *et al*. 2019). This model is congruent with Shenoy *et al*. (2012) who suggest SUMOylation is more likely to inhibit, rather than facilitate membrane localisation, and contrasts with Gonzales-Santamaria *et al*. (2012) who postulate SUMO binding facilitates membrane localisation through blocking its “closed” formation. This schematic is adapted from Carnero *et al*. (Carnero and Paramio 2014) Huang *et al*. (2012) and Lang *et al*. (2015) and made in Biorender.com with premade shapes.

5.4.3 PTEN, PTEN-3KR and AMPAR Expression

Figure 5.3.7.1. indicates that KD of PTEN significantly reduces surface GluA2. However, in a separate series of experiments investigating KD-rescue, the KD alone condition did not decrease surface GluA2 (Figure 5.3.9.1.). In these experiments, WT KD-rescue and PTEN-3KR conditions significantly increased GluA2 on the surface, supportive of the role of PTEN in GluA2 trafficking. The lack of replication of the PTEN KD decreasing GluA2 surface expression suggests this effect is not robust, and potentially sensitive to the culture conditions, plating density or network activity of the individual cell preparation. Nonetheless, taken with the observations that WT and PTEN-3KR overexpression increase surface GluA2, overall, my data support a role for PTEN role in positively regulating surface GluA2 expression.

Increased levels of PIP_3 is have been associated with synaptic plasticity (Arendt *et al*. 2010; Arendt *et al*. 2014; Jurado *et al*. 2010; Knafo *et al*. 2016). Indeed, Arendt *et al*. (2014) report that during LTP and LTD levels of PIP_3 are increased. Moreover, PTEN phosphatase activity is

necessary for LTD, and overexpression of WT, but not catalytically dead PTEN, can hamper AMPAR-mediated synaptic transmission under both basal and stimulation conditions (Jurado *et al*. 2010). It has been proposed that PTEN acts as a "switch" that dictates the outcome of this PIP_3 increase; there is an increase of PIP_3 in LTP and LTD which is counteracted by PTEN during LTD, blocking the net change in PIP_3 (Arendt *et al*. 2014). The effect is that the PTEN phosphatase activity favours LTD; although AMPAR surface expression was not examined directly, it is a well characterised outcome of LTD (Carroll *et al*. 1999; Fleming and England 2010; Jurado *et al*. 2010; Arendt *et al*. 2014).

Furthermore, PIP_3 can have a variety of effects on AMPARs, including limiting AMPAR surface expression (Arendt *et al*. 2010). PIP_3 is necessary to maintain AMPARs at synapses and inhibition of PIP_3 causes synaptic depression due to distribution from away from synapses to extra/ peri synaptic areas (Arendt *et al*. 2010). PIP_3 depletion has also been reported to increase AMPAR accumulation in spines and membrane (Arendt *et al*. 2010). Thus, an explanation of my data is that PTEN overexpression increases surface GluA2 through a mechanism that involves decreasing PIP_3 levels.

However, the relationship between PTEN, PIP_3, AMPAR trafficking and plasticity is complex, and previous research is contradictory. Imaging experiments would be useful to validate my finding that PTEN positively regulates surface GluA2. Considering Arendt *et al*. (2010) report that PIP_3 can regulate local AMPAR movement between synapses and extra/peri-synaptic regions, I would like to examine whether PTEN can influence this AMPAR movement through regulation of PIP_3. Arendt *et al*. (2010) propose that during basal conditions, PTEN maintains low PIP_3 levels in neurons.

It is noted that there was no significant difference in surface GluA2 expression between cells expressing PTEN KD-rescue WT and PTEN KD-rescue PTEN-3KR; both exhibited significantly increased surface GluA2 levels compared to control (scrambled PTEN shRNA).This was unexpected because PTEN-3KR has likely reduced phosphatase activity, evidenced by increased P-Akt (Figure 5.3.3.1.), so its lack of effect on surface GluA2 compared to WT argues against the involvement of PIP_3 levels in surface AMPAR expression. Nonetheless, PTEN-3KR does retain some phosphatase activity since it partially rescues the effect of PTEN KD. I propose that this remaining phosphatase activity is sufficient to limit potential PIP_3-depletion-Dependent increases to AMPAR levels. Furthermore, the residual phosphatase activity coupled with marked overexpression of PTEN constructs may account for the lack of difference between PTEN-WT and PTEN-3KR in these experiments. The experiment therefore should be repeated with lower expression levels of the rescue constructs.

5.5 Summary

In summary, the data I present suggest

- There is no significant difference in PTEN-3KR stability compared to PTEN-WT
- There is no significant difference in the ability of PTEN-3KR to dimerize with PTEN-WT
- PTEN-3KR is defective in its ability to inhibit the Akt pathway compared to PTEN-WT in HeLa cell lines
- P-Erk levels are not significantly altered by PTEN loss or expression of WT or PTEN-3KR
- PTEN may play a modest role in GluA2 surface expression in neurons
- Enhanced SUMOylation and Ubiquitination of PTEN on PTEN-3KR does not alter GluA2 surface expression

5.6 Conclusion

In this chapter I have characterised aspects of the phosphatase activity, stability and dimerization ability of PTEN-3KR. This has revealed that PTEN-3KR has reduced ability to regulate phospho-Akt. Given that PTEN-3KR is more SUMOylated than WT, this suggests enhanced SUMOylation may negatively regulate PTEN phosphatase activity, but more research is needed to clarify this.

The lack of reproducibility means that interpretation of the GluA2 surface biotinylation experiments must be treated with caution. However, in conjunction with evidence that increasing PTEN expression increases surface GluA2, my tentative conclusion is that PTEN positively regulates surface GluA2. This is in contrast to reports showing that upregulation of PTEN is associated with reduced surface GluA2 (Liu *et al*. 2018; Liu *et al*. 2013). A potential explanation for these apparent discrepancies is that PIP_3 is necessary for AMPAR clustering and surface expression (Arendt *et al*. 2010). Since PTEN negatively regulates PIP_3 (Maehama and Dixon *et al*. 1998), it is possible that PTEN exerts its effect on GluA2 through its activity against PIP_3. Arendt *et al*. (2010) postulate that under basal conditions, PTEN may limit PIP_3 levels in unstimulated neurons. Furthermore, it has been reported that PTEN counteracts PIP_3 increases after NMDA stimulation, blocking net change in PIP_3, skewing plasticity events towards LTD which is associated with reduction of AMPARs at the synaptic membrane (Arendt *et al*. 2014; Carroll *et al*. 1999; Fleming and England 2010).

With this in mind, it was unexpected that PTEN-3KR did not have a differential effect to WT, given that it had reduced activity against Akt activation, a known outcome of PIP_3 signalling (Rosivatz and Woscholski 2009). It would be useful to repeat this experiment with a lower level of expression, in case overexpression is confounding the result. Taken together, my current data suggest that enhanced SUMOylation and/or ubiquitination of PTEN do not influence its effect on surface GluA2.

5.7 Future Experiments

To extend these findings, the following experiments could be carried out

- Repeat surface biotinylation measuring surface GluA2 with expression of KD-rescue WT and PTEN-3KR at endogenous levels
- Repeat surface biotinylation measuring surface GluA2 with expression of KD-rescue catalytically inactive PTEN mutant, to confirm role of PTEN phosphatase activity in regulating surface GluA2. This could be validated by observing changes after PI3K inhibitor treatment.
- Assess plasticity capacity of WT, PTEN-3KR and catalytically inactive PTEN using *in vitro* LTD experiments.
- Examine P-Akt levels between PTEN KD, WT, PTEN-3KR and control conditions in neurons
- The model in Figure 5.4.1 could be directly tested by expressing WT versus catalytically inactive SENP1 in the PTEN HeLa cells lines and examining P-Akt levels, to establish whether it is enhanced SUMO on PTEN-3KR that reduces its ability to regulate Akt phosphorylation.
- Imaging experiments should be repeated with lower levels of PTEN-WT and PTEN-3KR expression, and compartment specific markers, to compare their localisation

6 PTEN, SUMO and Retromer

6.1 Introduction

6.1.1 PTEN and Retromer

Recent evidence suggests that PTEN may regulate protein trafficking via regulation of the retromer complex (Shinde and Maddika, 2017). Retromer sorts cargo proteins to be trafficked from endosomes to the Golgi and plasma membrane (Cullen and Korswagen, 2012; Seaman, 2012, Burd and Cullen, 2014). KD of retromer component SNX27 induces a loss of over 100 proteins from the cell surface, highlighting the importance of retromer in membrane protein trafficking (Steinberg *et al*. 2013). Through trafficking of neuronal proteins, retromer can regulate plasticity, and this process is thought to be perturbed in AD and PD (Munsie *et al*. 2015; Temkin *et al*. 2017; Choy *et al*. 2014; Wang *et al*. 2012).

PTEN may have a role in neuronal surface expression of GluA2 (Figures 5.3.7 and 5.3.10), which has also been reported previously (Moult *et al*. 2010; Liu *et al*. 2013). To extend my findings, I decided to test the role of PTEN in retromer mediated trafficking, partly due to evidence that GluA2 is reported to be a retromer cargo (Temkin *et al*. 2017). I reasoned that PTEN could therefore influence AMPAR trafficking through retromer. PTEN has been reported to interrupt formation of the retromer complex via direct interactions with SNX27, but this has not been widely tested (Shinde and Madikka, 2017). I was interested to see if PTEN can regulate retromer components and trafficking of other retromer cargoes. This will help to elucidate the role of PTEN in membrane protein trafficking, a process that is relevant to fundamental aspects of cellular regulation, as well as plasticity, neurodegeneration and cancer (Temkin *et al*. 2017; Munsie *et al*. 2015; Zhang *et al*. 2018).

Additionally, the role of SUMOylation of PTEN on retromer has not been examined. So far, I have shown PTEN-3KR is more SUMOylated and ubiquitinated than WT and has limited activity against the Akt pathway. I was interested to see if SUMOylation of PTEN has a role in its influence over retromer.

6.1.2 Importance of the Retromer Complex in Neurons and Synaptic Plasticity

Retromer is reported to be directly involved in plasticity and can regulate trafficking of neuronal receptors such as β2 adrenergic receptors (β2ARs), AMPARs and dopamine transporters (Munsie *et al*. 2014; Choy *et al*. 2014; Wu *et al*. 2017, Cai *et al*. 2011). Deficits in retromer are reported to perturb an array of neuronal processes including neuronal maturation, LTP, AMPAR trafficking, AMPAR-mediated transmission, NMDAR endocytosis and Aβ deposition and metabolism (Cai *et al*. 2011; McMillan *et al*. 2020; Wang *et al*. 2012;

Bhalla *et al*. 2012; Choy *et al*. 2014; Temkin *et al*. 2011; Temkin *et al*. 2017; Tian *et al*. 2015; Li, Chiu, and Pratico 2020) (See Introduction 1.4.6-1.4.7 for more detail on this). Notably, KD of retromer component VPS35 KD blocks LTP, through inhibition of AMPAR sorting and insertion at the membrane necessary for LTP (Temkin *et al*. 2017).

Table 7. Key Retromer Proteins (For More Information see General Introduction 1.4).

Protein	Key Mechanism of Action	Roles in Neuronal Regulation
SNX27	Adaptor protein, mediates interactions between cargoes and retromer (Burd and Cullen, 2014).	Regulates AMPAR trafficking, LTP, neurotransmission (McMillan *et al*. 2020). Regulates NR2C endocytosis, crucial for mouse survival past 3 weeks (Cai *et al*. 2011)
VPS26	Regulates cargo recognition and localisation, VPS35 membrane localisation, and VPS35-SNX protein interactions (Reddy and Seaman, 2001; Gokool *et al*. 2007; Fjorback *et al*. 2012).	Regulates VPS35 levels in neurons (Bhalla *et al*. 2012)
VPS29	Binds VPS35, stabilises VPS26 and VPS35 (Kovtun *et al*. 2018; Jimenez-Orgaz *et al*. 2018; Baños-Mateos, Rojas, and Hierro 2019; Fuse *et al*. 2015)	Regulates endo-lysosomal function and synaptic transmission (Ye *et al*. 2020)
VPS35	Cargo recognition (Burd and Cullen, 2014), major regulator of cell surface protein recycling (Steinberg *et al*. 2013).	Regulates Aβ deposition/ levels (Li *et al*. 2019; Small *et al*. 2005), AMPAR surface levels and spine maturation (Tian *et al*. 2015). Critical in LTP (Temkin *et al*. 2017).

Retromer dysfunction is implicated in a range of neurodegenerative diseases including AD and PD (Small *et al*. 2005; Muhammad *et al*. 2008; Bhalla *et al*. 2012; Munsie *et al*. 2014). Mutations of retromer genes are also found in AD and PD, and are thought to lead to deficits in both receptor trafficking and Amyloid metabolism (Small *et al*. 2005; Muhammad *et al*. 2008; Bhalla *et al*. 2012; Temkin *et al*. 2017; Munsie *et al*. 2014; see General Introduction 1.5.11-1.5.13). Haploinsufficiency of VPS35, a key retromer protein, is associated with AD (Wen *et al*. 2011), and levels of VPS26 and VPS35 are downregulated in AD brains (Small *et al*. 2005). Furthermore, VPS35 upregulation in Tau/APP mutant mice reduces Aβ

deposition and levels and improves behavioural deficits and neuroinflammation (Li *et al*. 2019).

6.1.3 Retromer, Phosphoinositides and PTEN

There is a potential role for PTEN phosphatase activity in retromer regulation, as PIP_3 levels are known to influence retromer function (Verges, Sebastian, and Mostov 2007; Laporte *et al*. 2002). PIP_3 levels are reported to influence SNX27 targeting to endosomes (Cai *et al*. 2011), and SNX1/2 localisation with VPS26 and membrane targeting is dependent on PIP_3 (Vergés *et al*. 2006). Therefore, it is plausible that the phosphatase action of PTEN could influence retromer mediated trafficking. This is worth exploring as AMPARs are also influenced by PIP_3 levels, and PTEN phosphatase activity plays a role in plasticity and has a preference for synaptic depression (Jurado *et al*. 2010; Arendt *et al*. 2010, Arendt *et al*. 2014; Knafo *et al*. 2016). Furthermore, PI3K signalling favours synaptic potentiation (Arendt *et al*. 2010). Therefore, PTEN's lipid phosphatase influence on retromer needs to be further explored, as this may elucidate its role in plasticity. Given than PTEN-3KR seems to have reduced ability to limit P-Akt (Figure 5.5.3), suggestive of reduced lipid phosphatase activity (Stambolic *et al*. 1998; Myers *et al*. 1998; Maehama and Dixon 1998), this makes a useful tool to examine the role of lipid phosphatase activity alongside KD and WT conditions, and can also enable testing of PTEN SUMOylation and ubiquitination in this system.

6.1.4 PTEN Direct Interactions and Retromer-Trafficking

Aside from the potential for PTEN phosphatase action to influence retromer, Shinde and Maddika (2017) showed that through interaction with SNX27, PTEN can influence retromer in the context of GluT1 trafficking (GluT1 can be used as a model cargo to study retromer, see 5.1.5.). PTEN can bind SNX27 independently of its catalytic activity, which blocks the ability of SNX27 to associate with VPS26, reducing retromer formation and forward-trafficking of GluT1 (Shinde and Maddika, 2017). Diminishment of PTEN levels increased GluT1 surface expression and glucose uptake. Expression of WT PTEN, but not a PTEN mutant unable to bind SNX27 (PTEN ΔTKV, which has a PDZ binding motif deletion) was able to rescue these effects. It was concluded that through binding to SNX27 and sequestering it, PTEN could interrupt retromer formation and influence trafficking of GluT1 away from Rab11-positive recycling endosomes, and move it towards lysosomes to be degraded, which was evidenced by GluT1 co-localisation with LAMP1 (Shinde and Maddika, 2017). This shows a direct, phosphatase-independent role for PTEN in retromer-mediated trafficking of GluT1 (Shinde and Maddika *et al*. 2017).

6.1.5 PTEN and Possible Regulation of Retromer Via TFEB

Transcription Factor EB (TFEB) is described as "*a master regulator of lysosomal pathways",* and is strongly implicated in lysosomal degradation in settings relevant to neurodegeneration (Palmieri *et al*. 2017; Palmieri, Pal, and Sardiello 2017; Sardiello *et al*. 2009; Settembre *et al*. 2012; Martini-Stoica *et al*. 2016). PTEN has been implicated in this through the proposed feedback loop of TFEB–PTEN–Akt–mTOR–TFEB, whereby through upregulation of PTEN by TFEB, Akt and mTOR are then inactivated, which further activates TFEB (Polito *et al*. 2014; Settembre *et al*. 2012). mTOR may not be crucial in this pathway as Palmieri *et al*. (2017) reported that Akt phosphorylates TFEB at S467 which increases its activity, and Akt inhibition can increase TFEB activity in an mTORC independent manner (Palmieri *et al*. 2017). Furthermore, this Akt inhibition-driven TFEB upregulation can enhance clearance of aggregate proteins in neurodegeneration (Palmieri *et al*. 2017).

TFEB is implicated in Aβ plaque pathology in AD by increasing autophagasome formation and lysosomal fusion; its expression can also increase the uptake, trafficking, and lysosomal degradation of Aβ42 in primary astrocytes (Xiao *et al*. 2014; Settembre *et al*. 2012; Martini-Stoica *et al*. 2016), and can also regulate several Tau species (Polito *et al*. 2014). Polito *et al*. (2014) reported that TFEB interacts with the *PTEN* gene through two Coordinated Lysosomal Expression and Regulation (CLEAR) -containing sequences in its promoter; CLEAR sequences exist in many genes in the lysosomal pathway and are bound by TFEB, with an effect of upregulating their transcription (Sardiello *et al*. 2009). TFEB expression increases PTEN expression *in vitro* and *in vivo;* as well as PTEN levels, TFEB levels correlated with reduced PHF1-positive Tau levels (Polito *et al*. 2014). As the availability of PTEN regulated the ability of TFEB to reduce certain phospho-Tau species, it was concluded that PTEN is targeted by TFEB, which is necessary for TFEB-mediated Tau clearance (Polito *et al*. 2014). This was also reflected in behavioural experiments in rTg4510 Tauopathy mice; learning and memory outcomes were improved by TFEB expression shown in Morris water maze tests (Polito *et al*. 2014). PTEN KD reduced TFEB-mediated alleviation of pTau, suggesting PTEN is critical in TFEB's ability to clear pTau (Polito *et al*. 2014). Polito *et al*. (2014) suggest that upregulation of TFEB may improve aggregate protein clearance by enhancing autophagasome clearance.

Recently, it was discovered that TFEB overexpression can upregulate RNA and total protein levels of retromer components SNX27, VPS26 and VPS35 (Curnock *et al*. 2019). Therefore,

I hypothesized that by indirectly upregulating TFEB via P-Akt suppression, PTEN could upregulate retromer components levels via TFEB, according to studies by Polito *et al*. (2014) and Curnock *et al*. (2019). In summary, PTEN is a target of TFEB, and also can indirectly cause its activation (Polito *et al*. 2014); this is relevant to neurodegeneration as TFEB has a role in aggregate protein clearance (Palmieri *et al*. 2017; Polito *et al*. 2014), and can even directly influence retromer components which are also involved in protein clearance (Curnock *et al*. 2019; Vagnozzi and Pratico 2019; Ansell-Schultz *et al*. 2018).

6.1.6 GluT1 and ASCT2 as Model Retromer Cargoes in Cell Lines

GluT1 trafficking is known to be influenced by PTEN (Shinde and Maddika, 2017); in addition, I tested the effect of PTEN of trafficking of another retromer cargo: Alanine, Serine, Cysteine Transporter 2 (ASCT2). GluT1 and ASCT2 are both regulated by both SNX27 and VPS35 (Steinberg *et al*. 2013; Scalise *et al*. 2018; Yang *et al*. 2018; Curnock *et al*. 2019) and can therefore be used as retromer model cargos to examine the effects of PTEN and PTEN SUMOylation on retromer in cell lines.

ASCT2 surface localisation is necessary for glutamine influx and efflux, and has increased expression in proliferative cells (Console *et al*. 2015; Yang *et al*. 2018; Scalise *et al*. 2018; Bhutia and Ganapathy 2016). Previous research in ASCT2 trafficking has identified a role for SNX27 (Steinberg *et al*. 2013; Yang *et al*. 2018). Knockout (KO) of SNX27 in cell lines reduces surface ASCT2 levels, reducing glutamine uptake, which leads to restriction of cellular proliferation (Yang *et al*. 2018). Yang *et al*. (2018) suggest ASCT2-SNX27 interactions through the SNX27 PDZ domain are important in rescuing ASCT2 from lysosomal degradation, reducing this interaction through SNX27 KO leads to increased ASCT2 degradation and downstream effects on autophagy due to mTORC1 dysregulation. VPS35 KO HeLa cells, ASCT2 is lost from the surface and colocalises with LAMP1-positive endosomes and lysosomes significantly more, suggesting enhanced lysosomal degradation during retromer depletion (Curnock *et al*. 2019). This effect was rescued by GFP-VPS35 expression in the KO cells, which restored ASCT2 surface levels. ASCT2 is found to be upregulated in various types of cancer and is a pharmacological cancer target; since its inhibition can block cancer cell growth (van Geldermalsen *et al*. 2016; Wang *et al*. 2015; Jeon *et al*. 2015). ASCT2 activity is critical in growth of basal-like breast cancer cells *in vitro* (van Geldermalsen *et al*. 2016). Furthermore, in mice which had undergone xenograph of breast cancer HCC1806 cells, shRNA KD of ASCT2 in these cells was associated with enhanced survival and reduced tumour growth (van Geldermalsen *et al*. 2016). GluT1 is also relevant in cancer through its effect on glucose uptake regulation (Morani *et al*. 2014; Oh *et al*. 2017).

PTEN's role in trafficking of ASCT2 has not been examined, neither has the role of SUMOylation of PTEN in retromer mediated trafficking. Testing this will help to improve understanding of whether effects seen previously on GluT1 trafficking are restricted to this cargo, or whether PTEN affects retromer trafficking more widely, and if PTEN SUMOylation is involved.

6.2 Aims

The purpose of this chapter is to assess the role of PTEN in retromer mediated trafficking, by testing which cargoes are involved and how PTEN may influence retromer components. I also wanted to compare PTEN-WT and PTEN-3KR in these contexts to establish whether PTEN SUMOylation and/or ubiquitination is involved.

Specifically, my aims were

- To establish how PTEN may influence retromer-mediated trafficking
- To examine which retromer cargoes are influenced by PTEN
- To establish whether SUMOylation and/or ubiquitination is are involved in retromer-mediated trafficking and regulation by comparing PTEN-WT and PTEN-3KR effects in these contexts

6.3 Results

6.3.1 PTEN-WT and PTEN-3KR Show Differential Binding to SNX27

It has recently been shown that PTEN directly interacts with SNX27 and is able to sequester it and block retromer formation (Shinde and Maddika, 2017). To determine whether the enhanced SUMOylation or ubiquitination could influence this interaction I investigated the association of WT and PTEN-3KR with SNX27 (Figure 6.3.1.1). Therefore I probed for SNX27 on Western blots from the experiments shown in Figure 5.3.8, where I expressed either GFP, GFP-tagged WT-PTEN or 3KR-PTEN alongside Myc-PSD-95 in HEK cells, then carried out a GFP trap (see methods for full protocols). Compared to PTEN-WT, PTEN-3KR bound significantly less SNX27, suggesting SUMOylation and/or ubiquitination on PTEN may interfere with the interaction.

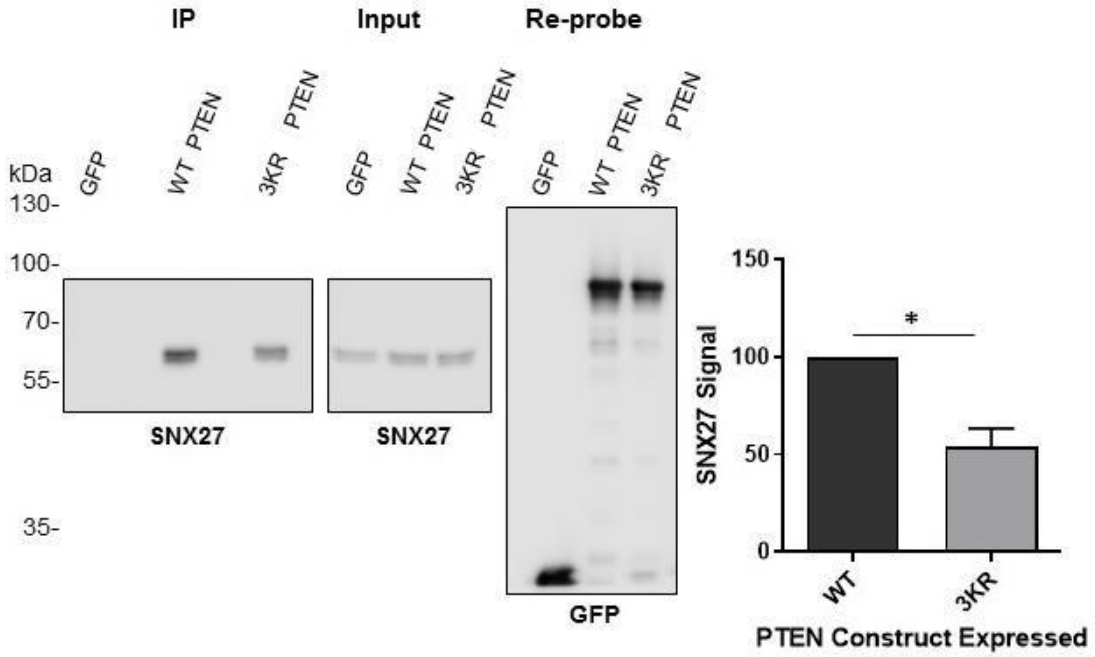


Figure 6.3.1.1. PTEN-WT interacts with SNX27 significantly more strongly than PTEN-3KR.

Representative blot showing immunoprecipitated endogenous SNX27 in HEK293T cells, probed on the same blots as in Figure 5.3.8. Cells were transfected with Myc-tagged PSD-95 along with GFP, PTEN-WT or PTEN-3KR and subject to immunoprecipitation and Western blot protocols as in Methods 3.4.1. Samples were blotted for SNX27 and GFP. SNX27 signal was normalised to GFP, PTEN-3KR was compared to WT set to 100 as a control. PTEN-3KR was then compared with WT which was set to a hypothetical value of 100. A one sampled T-test was then used to establish significance ($p=0.0372$, $N=3$). Re-probe is of immunoprecipitation.

6.3.2 Expression of WT or PTEN-3KR does not Alter SNX27-VPS26 Interaction

Previous research shows that PTEN can sequester SNX27, blocking its association with VPS26 (Shinde and Madikka, 2017). As PTEN-3KR bound SNX27 less than WT (Figure 6.3.1.1), I hypothesised that PTEN-3KR may interrupt SNX27/VPS26 association less than PTEN-WT. To test this, and whether overexpression would influence SNX27/VPS26 association in my hands, I immunoprecipitated GFP-SNX27 and blotted for VPS26, alongside either TAP, TAP-PTEN-WT or TAP-PTEN-3KR in HEK293T cells (Figure 6.3.2.1). Although interpreted with caution due to N=2, no effect was seen of expression of WT or PTEN-3KR on the amount of VPS26 immunoprecipitated by SNX27, so overexpression of PTEN seems unlikely to affect VPS26-SNX27 interactions in this context.

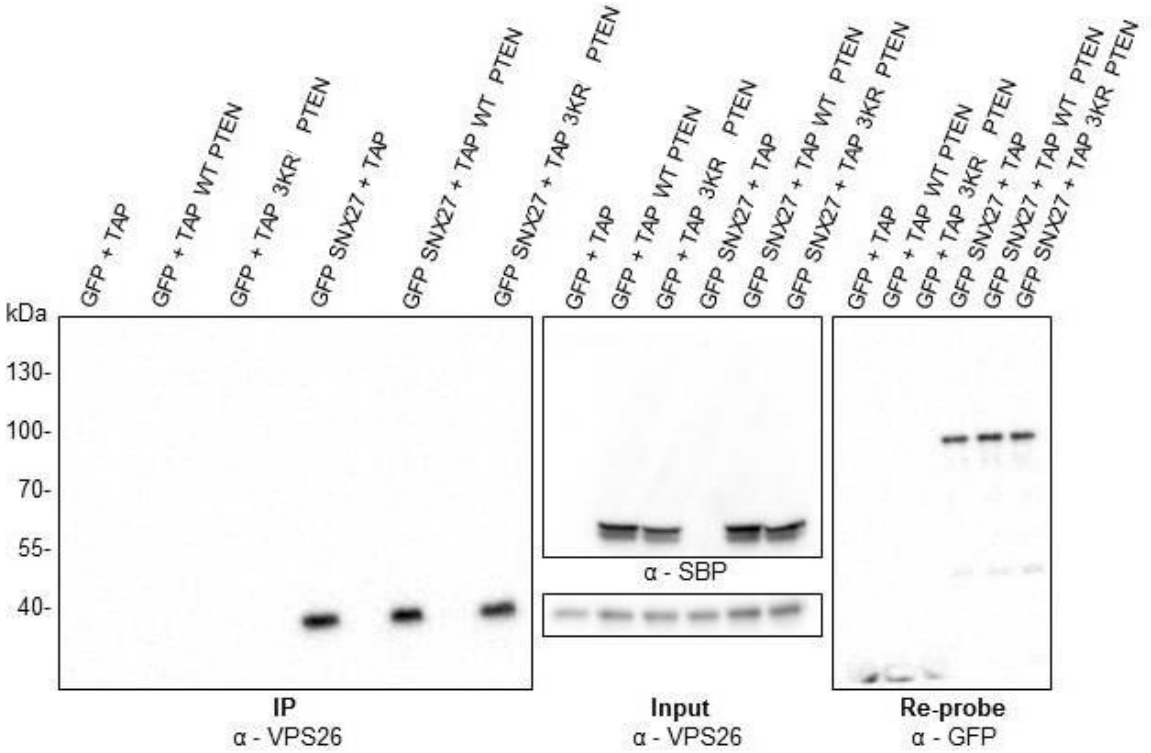


Figure 6.3.2.1. Expression of PTEN-WT or PTEN-3KR does not alter SNX27-VPS26 interaction in HEK cells.
Representative blot of endogenous VPS26 immunoprecipitated by GFP-SNX27. HEK293T cells were transfected with either empty GFP plasmid or GFP-SNX27 plasmid, along with empty TAP plasmid, TAP-WT-PTEN or TAP-3KR-PTEN. Two days later, cells were lysed and immunoprecipitated with GFP beads. Samples were then subject to Western blot and blotted for GFP, SBP and VPS26 (N=2).

6.3.3 PTEN-WT Expression, but not PTEN-3KR, can Alter Retromer Component Levels

After finding differential effects of PTEN-WT and PTEN-3KR in terms of SNX27 binding, I investigated whether levels of retromer components would change between KD, WT and PTEN-3KR conditions. SNX27 and VPS26 are vital components of the retromer complex; their individual or simultaneous knockdown causes a variety of proteins to be lost from the cell surface (Steinberg *et al*. 2013). I was therefore interested in whether PTEN could influence these proteins, given that PTEN can bind SNX27 (Shinde and Maddika, 2017). Furthermore, there is the potential for PTEN to influence total levels of SNX27, VPS26 and VPS35, through TFEB (see 6.1.5.1) (Polito *et al*. 2014; Curnock *et al*. 2019).

I therefore blotted for SNX27, VPS26 and VPS35 in lysates from my PTEN HeLa cell lines (Figure 6.3.3.1), which I have previously characterised in terms of PTEN, p-Akt, Akt and p-Erk expression (Figures 5.3.3-5.3.4). I saw that VPS26 and SNX27 were both significantly increased in cells expressing PTEN-WT compared to Scr, PTEN KD or PTEN-3KR. VPS35 levels did not change significantly between conditions. These results suggest that VPS26 and SNX27 are either being degraded more slowly, or that their transcription is upregulated in the PTEN-WT expressing HeLa cell lines compared to Scr, PTEN KD or PTEN-3KR expressing cells. This supports my hypothesis, that phosphatase active PTEN-WT can drive retromer component upregulation, possibly via TFEB (Polito *et al*. 2014; Curnock *et al*. 2019).

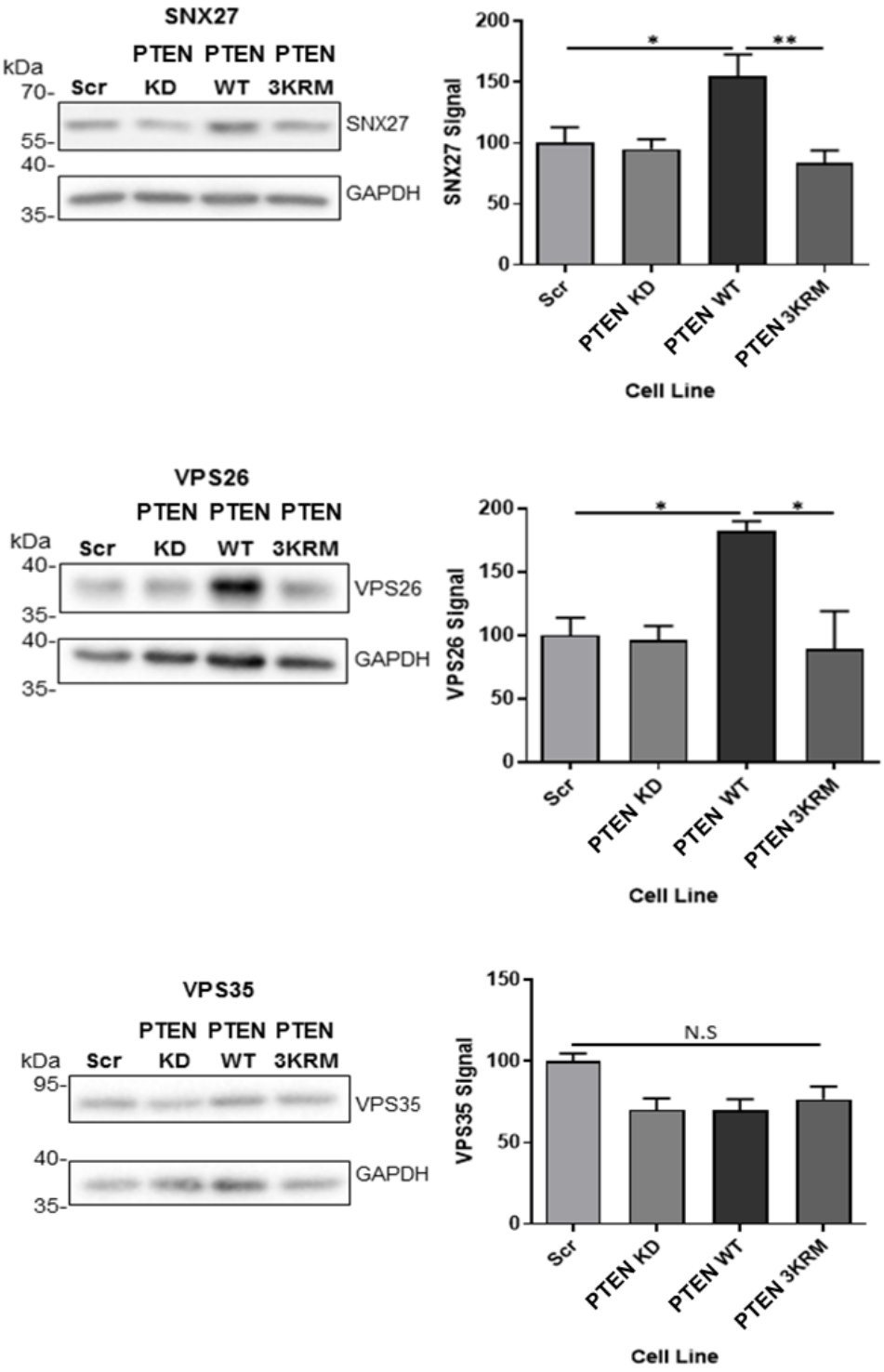


Figure 6.3.3.1. PTEN-WT overexpression, but not PTEN-3KR overexpression, significantly increases SNX27 and VPS26 levels in HeLa cells.

Samples from HeLa cell lines shown in Figure 4.3.2 were subject to Western blot protocols and blotted for SNX27, VPS26 and VPS35 then stripped and blotted for GAPDH. A, C and E) show representative Western blots of SNX27, VPS26 and VPS35. B, D and F) show a quantification of each protein. SNX27 levels were normalised to GAPDH. KD, WT and PTEN-3KR were then normalised to Scr as a control. All experiments were individually normalised to mean of each experiment, then a one-way ANOVA with Tukey correction was used to analyse significance (SNX27 N=4, VSP26 N=3, VPS35 N=3; *=$p \leq 0.05$, **=$p \leq 0.01$).

6.3.4 SNX27 Levels are not Altered in PTEN KD, WT or PTEN-3KR Expressing Neurons

Due to evidence showing that the retromer complex is involved in AMPAR trafficking (Temkin *et al*. 2017), evidence showing that PTEN may disrupt retromer formation (Shinde and Maddika, 2017), and my data showing PTEN can affect SNX27 levels in HeLa cells (Figure 6.3.3.1), SNX27 levels were blotted in the total samples in Figure 5.3.9.1, where I expressed PTEN-KD, PTEN-WT and PTEN-3KR Lentivirus in neurons. There was no significant difference in SNX27 level between any of the conditions, suggesting any effects seen on GluA2 in Figures 5.3.7.1 and 5.3.10.1 are not due to changes in SNX27 total levels.

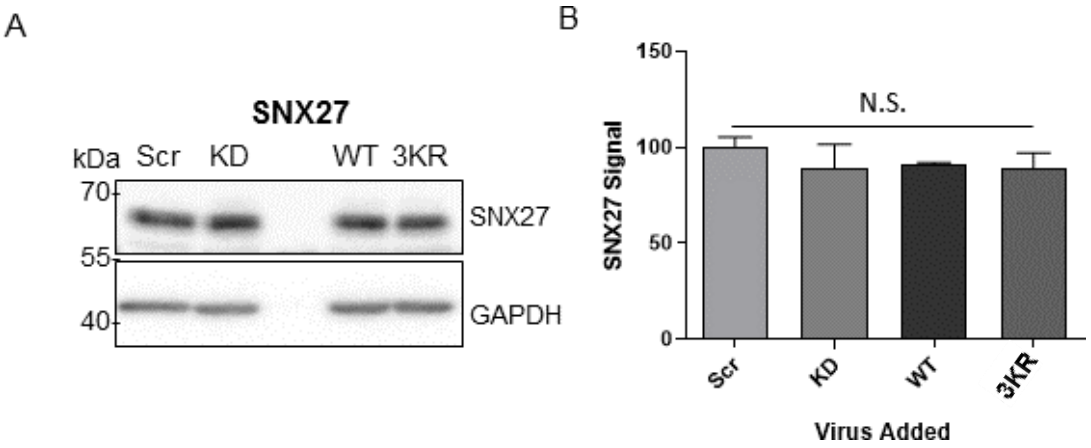


Figure 6.3.4.1. Lentiviral PTEN KD and overexpression of WT or PTEN-3KR do not significantly alter total SNX27 levels in neurons.
A) Representative blot showing SNX27 levels in surface biotinylation total samples shown in Figure 5.3.9.1. Neurons were left for 6/7 days after being infected with Scr, PTEN KD , PTEN-WT or PTEN-3KR Lentivirus on DIV 14/15. B) Graph showing quantification of SNX27. SNX27 was normalised to GAPDH. Samples were normalised to Scr and all experiments were individually normalised to the mean of each experiment. One-way ANOVA analysis was then carried out with Tukey correction. There were no significant differences between any conditions (N=3).

6.3.5 PTEN-3KR Can Influence GluT1 Trafficking

PTEN can regulate GluT1 surface expression through binding with SNX27 (Shinde and Madikka, 2017). My results indicate that PTEN-3KR binds to SNX27 less than PTEN-WT (Figure 6.3.1.1.). Therefore, I next tested if PTEN-3KR expression would alter GluT1 surface levels compared to WT. I therefore surface biotinylated the Scr/PTEN-KD/PTEN-WT/PTEN-3KR HeLa cell lines (previously characterised for PTEN levels in Figure 6.3.5.1.), followed by Western blotting for GluT1 (Figure 6.3.5.1.A/B).

GluT1 surface expression was significantly lower in PTEN-3KR expressing cells, compared with Scr. PTEN-3KR expression therefore seems to have a gain of function effect of reducing surface GluT1. PTEN KD, or PTEN-WT overexpression had no significant effect compared to Scr on surface GluT1 levels, in contrast to previous reports (Shinde and Maddika, 2017). It is noted that the PTEN-3KR and WT cells vastly overexpressed PTEN compared to the level of endogenous PTEN seen in Scr (Figure 6.3.5.1.). Total GluT1 levels were not significantly altered between any of the conditions.

The results of the surface biotinylation are supported by preliminary evidence from confocal imaging carried out by Dr Ash Evans (Figure 6.3.5.1.C). Transfection of HeLa cells with GFP-tagged PTEN-WT and PTEN-3KR constructs, and subsequent staining for GLUT1, LAMP1 and PTEN, followed by confocal imaging shows GluT1 appears to be modestly reduced from cell surface in PTEN-3KR cells compared to WT. Furthermore, in PTEN-3KR cells, there appears to be more co-localisation between GluT1 and LAMP1, a lysosomal marker, suggesting GluT1 may not be being efficiently rescued from lysosomal sorting in this condition (although it is noted that total GluT1 levels do not differ significantly in the surface biotinylation samples (Figure 6.3.5.1.B).

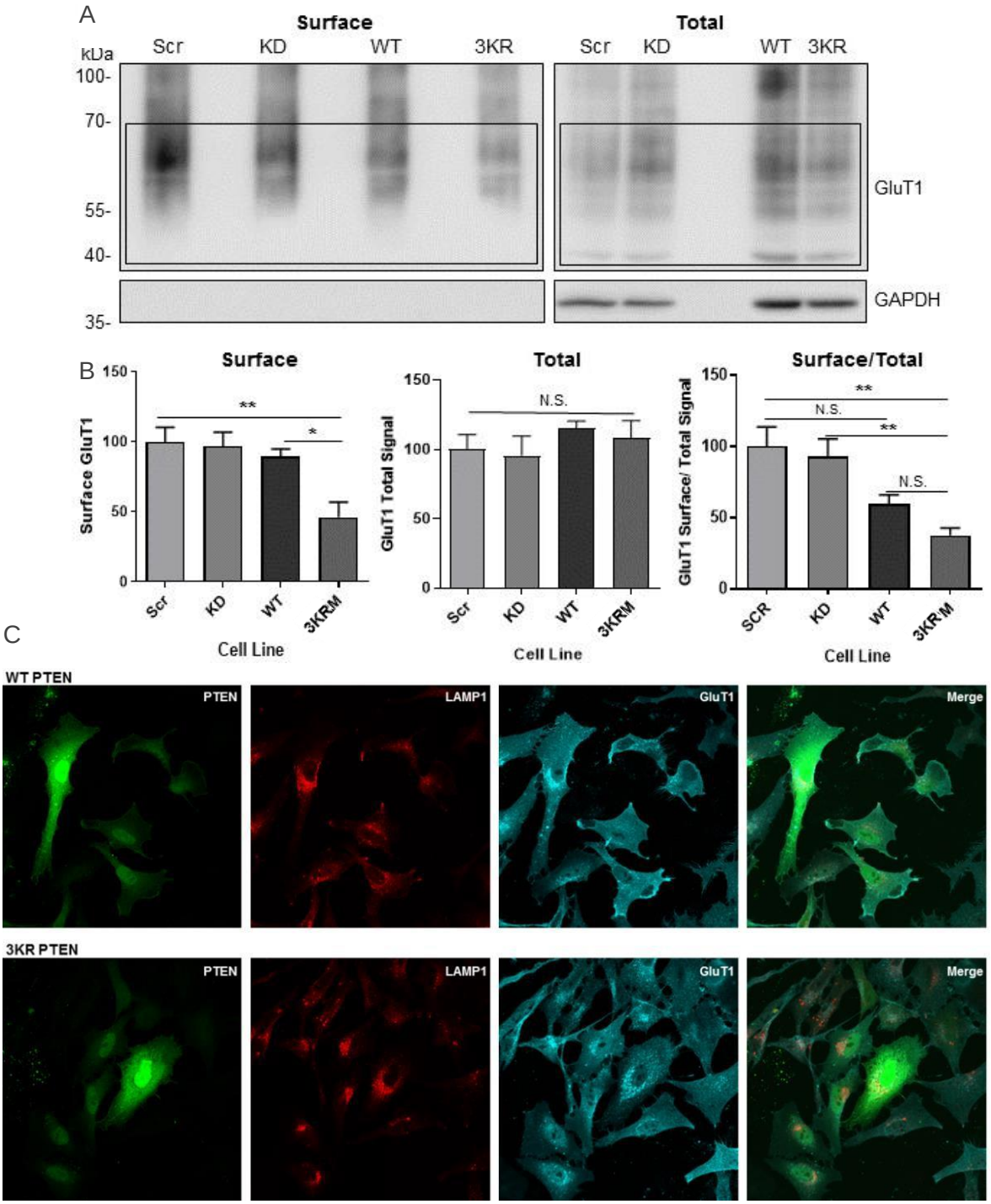


Figure 6.3.5.2. Surface GluT1 is Significantly Reduced in PTEN-3KR Expressing HeLa Cell Lines.

A) Representative blot of surface biotinylation of HeLa stable cell lines. HeLa cells expressing Scr, PTEN KD, PTEN-WT and PTEN-3KR were plated into wells of a 6-well dish, the next day cells were subject to surface biotinylation and blotted for GluT1. B) Quantification of A. Graphs show surface GluT1, Total GluT1 (normalised to GAPDH) and surface/total GluT1. Signals within individual experiments were normalised to the mean of that experiment, and One-way ANOVA with Tukey correction was used for statistical analysis (N=5, *=$p \leq 0.05$, **=$p \leq 0.01$). C) Confocal imaging by Dr Ash Evans of HeLa cells transfected with GFP PTEN-WT or PTEN-3KR and stained for GluT1, LAMP1 and PTEN (N=1).

6.3.6 PTEN is not involved in ASCT2 or TfR Trafficking

To determine whether the PTEN-3KR effect of reducing surface GluT1 is specific for GluT1 or affects other retromer cargoes, I repeated this experiment and blotted for another retromer cargo: Alanine, Serine, Cysteine Transporter 2 (ASCT2) (Yang *et al*. 2018) (Figure 6.3.6.1). I also added an extra condition: PTEN-C124S mutant (cloned by Dr K Wilkinson), which is catalytically inactive against PIP_3 (Maehama and Dixon, 1998). I surface biotinylated another batch of the HeLa cell lines with the addition of cells expressing PTEN-C124S. Samples were also blotted for Transferrin Receptor 1 (TRF1), which has not been reported as a retromer cargo. I reasoned that this would help determine whether the effect of PTEN-3KR is limited to retromer cargoes, or a more general effect on surface protein trafficking.

There was no significant difference between surface levels of ASCT2 or TfR1 between conditions, although there was a modest but significant decrease in total ASCT2 levels between Scr and the PTEN-C124S mutant. Therefore, my data indicate that the effect of PTEN-3KR in reducing GluT1 surface levels is not a general effect on retromer cargoes, or on surface proteins in general, but may instead be specific to GluT1, or affect a restricted subset of retromer cargoes.

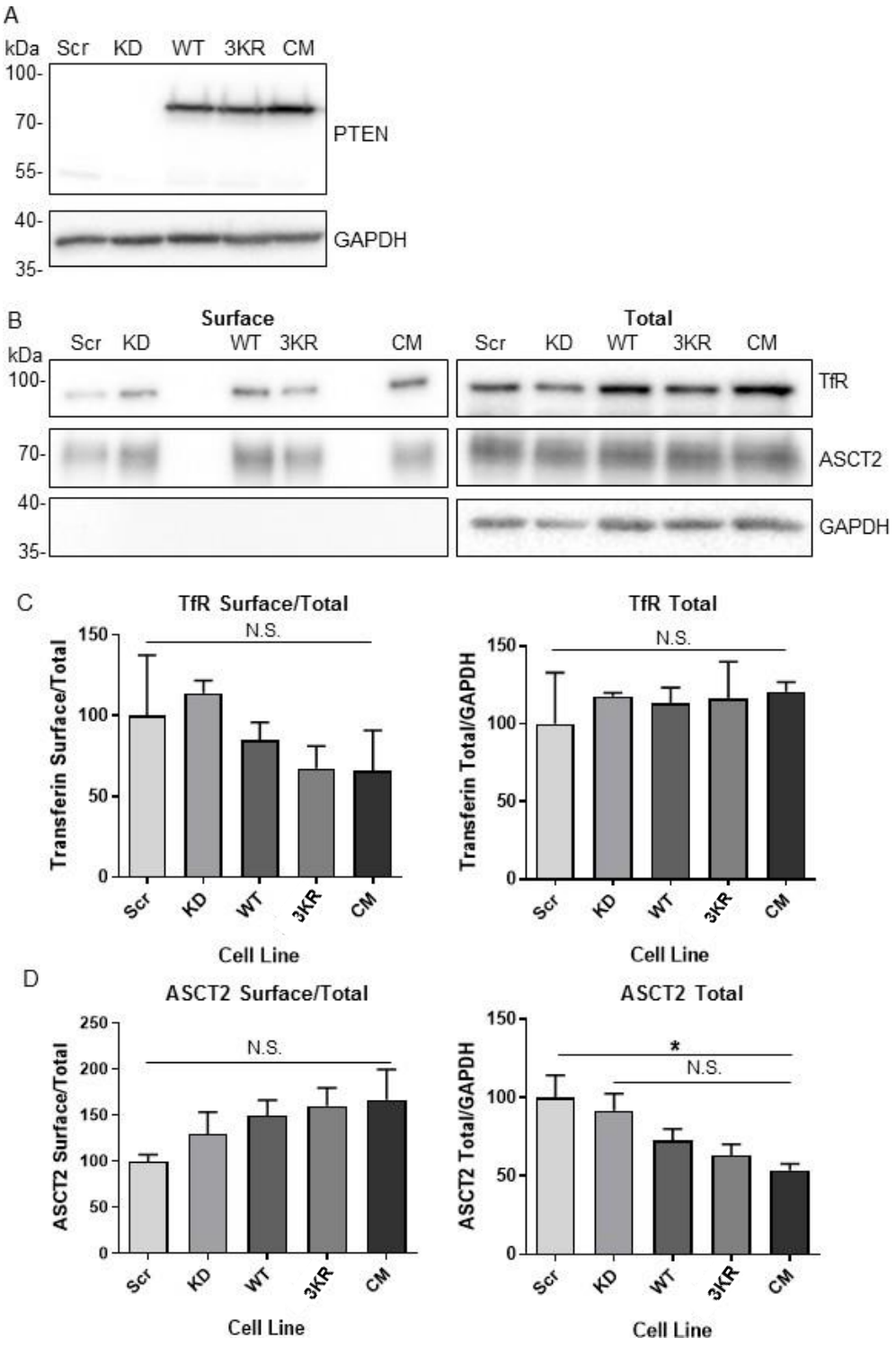


Figure 6.3.6.1. Surface ASCT2 and Transferrin Receptor levels are not significantly altered in PTEN KD, WT and mutant cell lines.

A) Blot showing level of PTEN expression in Scr, PTEN KD, PTEN-WT, PTEN-3KR and PTEN-C124S (CM) HeLa cell lines. B) Representative blot of surface biotinylation of HeLa cell lines. Cells were plated into wells of a 6-well dish, the next day, cells underwent surface biotinylation (see Methods 3.4.8) followed by Western blotting for ASCT2, Transferin receptor, and GAPDH. C/D) Quantification of surface and total signal of blot on the Left. Surface was normalised to total, and total was normalised to GAPDH. One-way ANOVA with Tukey correction was used to determine statistical significance (*=p ≤ 0.05, N=3). Signals within individual experiments were normalised to the mean signal from that experiment.

6.4 Discussion

In this chapter, I have shown that PTEN-3KR acts differently to PTEN-WT in:

- binding with SNX27 in HEK293T cells
- affecting expression of retromer components VPS26 and SNX27 in HeLa cells
- regulating GluT1 surface expression in HeLa cells

I have also shown that PTEN-WT or PTEN-3KR expression does not affect surface expression of another retromer cargo, ASCT2.

6.4.1 PTEN-SNX27 and VPS26-SNX27 Binding

PTEN-3KR bound significantly less than PTEN-WT to SNX27 (Figure 6.3.1.); it is not clear why this is, as PTEN is thought to bind SNX27 via its C-terminal PDZ binding motif (Shinde and Maddika, 2017), and mutations in PTEN-3KR are not near the PDZ ligand. As previous work has shown PTEN-SUMO site mutants localise differently (Huang *et al*. 2012), a possibility is that PTEN-3KR could localise differently and co-localise less with SNX27 than WT. Due to evidence that PTEN can sequester SNX27 and interrupt the SNX27-VPS26 association (Shinde and Maddika, 2017), I wanted to test whether overexpression of PTEN could do this in my samples. I hypothesised that PTEN-WT overexpression may limit SNX27-VPS26 interaction, but PTEN-3KR may not, as it bound SNX27 less. However, no obvious effect was seen after overexpression WT or PTEN-3KR on the amount of VPS26 immunoprecipitated by SNX27. It is noted the experiment was only carried out twice, although no obvious change was seen. It could be that lack of affect is an artefact of overexpression, in that increasing expression of both constructs increases binding to SNX27, and overshadows any difference in effect that could be seen at endogenous levels. Future experiments could include repeating the experiment at lower levels of PTEN expression.

6.4.2 PTEN and Regulation of Retromer Components in HeLa Cells and Neurons

The finding that PTEN-WT overexpression increases VPS26 and SNX27 levels is novel (Figure 6.3.3). PTEN can reportedly sequester SNX27 away from VPS26 (Shinde and Maddika 2017) and PTEN-3KR binds less to SNX27 less than PTEN-WT (Figure 6.3.1), so a possibility is that cells increase total levels of retromer components to compensate for SNX27 sequestration when PTEN-WT is expressed.

Due to recent evidence that TFEB can enhance expression of these proteins (Curnock *et al*. 2019), and that PTEN may indirectly upregulate TFEB through suppression of P-Akt (Polito *et al*. 2014), it could be that PTEN overexpression is causing this increase in retromer component levels through TFEB. Unlike PTEN-WT, PTEN-3KR does not increase SNX27 and VPS26 levels, and PTEN-3KR also lacks ability to supress P-Akt (Figure 6.3.3.1). This supports the notion that PTEN's phosphatase activity against P-Akt could upregulate retromer levels though TFEB activation (Figure 6.4.1.1). It is noted that components don't go down under PTEN-3KR conditions; this could be because the regulation of retromer components by PTEN is "off" under normal conditions, and only switched on when PTEN has higher expression or activation.

It is of interest that PTEN levels are reduced and P-Akt is increased in AD brains (Griffin *et al*. 2005). PTEN-driven upregulation of TFEB activity enhances clearance of AD related protein aggregates such as P-Tau, and subsequently upregulates PTEN (Polito *et al*. 2014). Taking these together, it could be that in AD, loss of PTEN blocks enhancement of TFEB activity through the PTEN-P-Akt-TFEB feedback loop, reducing the ability of cells to clear aggregate proteins (Polito *et al*. 2014). The retromer complex could also be involved, as it is suggested to enable protein clearance and be dysregulated in neurodegeneration (Mecozzi *et al*. 2014; Ansell-Schultz *et al*. 2018).

SNX27 levels were unchanged in Scr/ PTEN-KD/ PTEN-WT/ PTEN-3KR expressing neurons (Figure 6.3.4). This suggests SNX27 total levels are not involved in the changes to AMPAR trafficking observed upon perturbing PTEN levels (Figures 5.3.7.1. and 5.3.10.1). However, more experiments are needed to establish if PTEN-SNX27 binding occurs in neurons, and whether this is relevant to neuronal processes, or if other retromer components such as VPS26 are being influenced by PTEN in neurons.

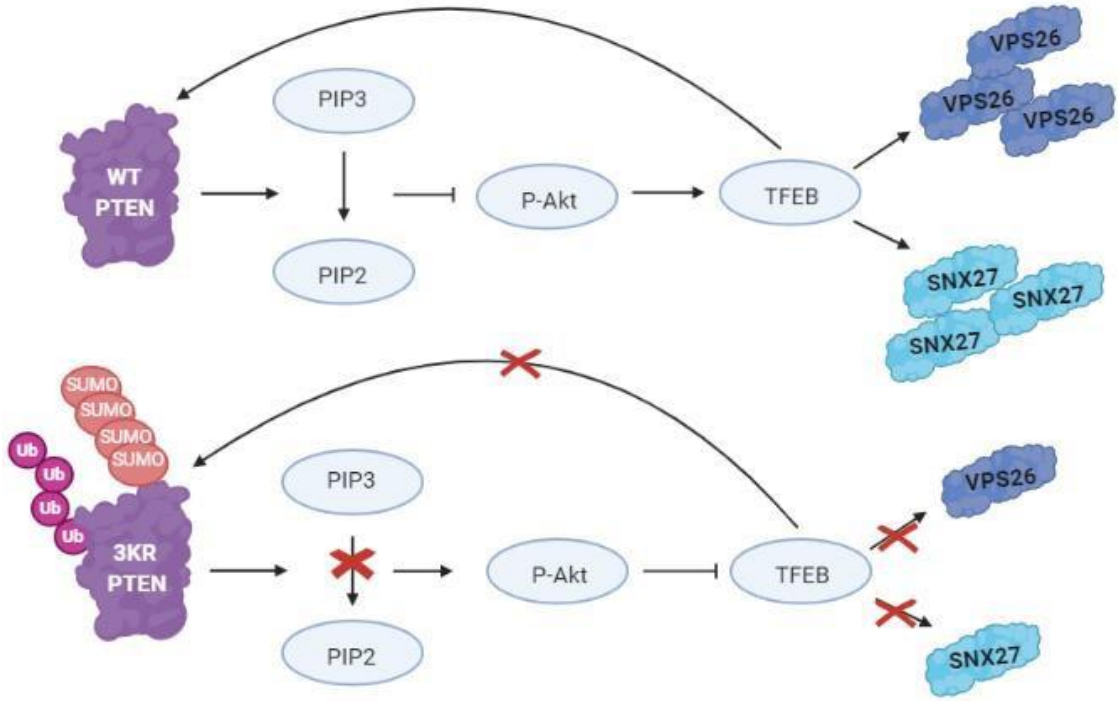


Figure 6.4.2.1. Schematic showing Regulation of Retromer Components SNX27 and VPS26 by PTEN.

This schematic shows that through dephosphorylation of PIP_3, PTEN-WT supresses P-Akt, which activates TFEB and upregulates VPS26 and SNX27. PTEN-3KR is unable to supress P-Akt, so TFEB activity is supressed and SNX27 and VPS26 are not increased. This model is based on work by Curnock *et al*. (2019) who demonstrated TFEB can upregulate retromer components, and Polito *et al*. (2014) who suggest PTEN can upregulate TFEB through suppression of P-Akt, which in turn upregulates PTEN. PTEN's effect of negatively regulating P-Akt through dephosphorylation of PIP_3 is well characterised (Maehama and Dixon 1998; Stambolic *et al*. 1998; Myers *et al*. 1998; Myers *et al*. 1997). Created in Biorender.com with premade shapes.

6.4.3 PTEN Effects on GluT1 and ASCT2 Trafficking

From results published by Shinde and Maddika (2017), I expected that surface GluT1 would be increased under PTEN KD and decreased under WT overexpression, given that PTEN sequesters SNX27 and limits GluT1 recycling. Correspondingly, given that PTEN-3KR binds less to SNX27 than PTEN-WT, I also expected surface GluT1 to be increased in PTEN-3KR expressing cells compared to PTEN-WT cells. However, no effect on surface GluT1 was seen in KD or PTEN-WT conditions compared to Scr, and surprisingly, PTEN-3KR reduced surface GluT1. PTEN-3KR therefore appears to have a gain of function effect of reducing GluT1 surface expression. This is not due to downregulation of SNX27 or VPS26 levels, however, when PTEN-WT is overexpressed, VPS26 and SNX27 are increased, which could compensate for the increase in PTEN-WT sequestration of SNX27. As retromer components

are not increased by PTEN-3KR, it's possible that reduced surface GluT1 is due to lack of this compensation.

These experiments suggest PTEN-3KR differs from PTEN-WT in regulation of GluT1 surface expression, regulation of retromer components and regulation of PTEN-SNX27 binding, possibly through enhanced levels of SUMOylation and/or ubiquitination. An important potential confounding issue is that PTEN-3KR lacks phosphatase activity against P-Akt (Figure 6.3.3). Therefore, it is difficult to ascertain whether its differences in activity compared to WT are due to enhanced SUMOylation and/or ubiquitination, or a lack of phosphatase activity. Regarding ASCT2, PTEN did not have an influence on surface levels (Figure 6.3.5.). This suggests that PTEN does not have the same effects across all retromer cargoes. It may be that in the case of GluT1, PTEN-SNX27 binding can disrupt forward trafficking through disruption of retromer formation (Shinde and Maddika *et al*. 2017), but this mechanism does not influence ASCT2.

6.5 Conclusion

In short, this chapter suggests that PTEN-3KR differs to PTEN-WT in its ability to regulate GluT1 trafficking, retromer component levels and in its binding to SNX27. It is possible that these effects are due to enhanced SUMOylation and/or ubiquitination of PTEN-3KR seen in Chapter 4, however, more research is needed to establish whether it is in fact SUMOylation or ubiquitination that is having this effect, or whether it is due to another mechanism acting on the PTEN-3KR. SUMOylation aside, these experiments suggest that PTEN regulates retromer function via increases in VPS26 and SNX27 (Figure 6.3.3), possibly through an indirect effect of PTEN in enhancing TFEB activity (Polito *et al*. 2014), as TFEB has been show to regulate VPS26 and SNX27 levels (Curnock *et al*. 2019).

6.6 Future Directions

- Compare VPS35 and VPS26 levels in PTEN HeLa cell lines after treatment with WT versus catalytically dead SENP, to establish if PTEN-3KR effects on these proteins are due to enhanced SUMOylation of PTEN. This could also be carried out with a DUB to investigate the effect of PTEN ubiquitination in this setting.
- Surface biotinylate PTEN HeLa cell lines after PI3K inhibitor treatment and examine surface GluT1. This will help to establish whether PTEN-3KR has its effects on surface GluT1 through deficits in Akt pathway regulation.

- Mass spectrometry of surface fraction of PTEN cell lines to establish if other surface proteins are affected by PTEN modulation. Further modulation of retromer components can then be used to establish if retromer is involved in this.
- Blotting TFEB and dephosphorylated (activated) TFEB to confirm if TFEB is activated by PTEN overexpression in the HeLa cell lines. RT-PCR could be used to establish if SNX27 and VPS26 are transcriptionally upregulated. Also, repeat blotting for SNX27 and VPS26 in cell lines with TFEB KD/inactivation to confirm a TFEB-Dependent role for PTEN in upregulating SNX27 and VPS26.

General Discussion

7 General Discussion

7.1 Context and Summary of Findings

7.1.1 PTEN, SUMO and Receptor Trafficking

The goal of this thesis was to further characterise the role of SUMOylation in PTEN regulation. I also aimed to understand the role of PTEN and PTEN-SUMOylation in cell-surface protein trafficking, a process fundamental to synaptic plasticity and relevant in AD (Chang *et al*. 2006; Shi *et al*. 2001; Passafaro *et al*. 2001). The finding that mutation of K254/K266/K289 to arginine (PTEN-3KR) increases SUMOylation and ubiquitination was novel, and contrasts with reports showing that single/ individual mutation of these sites reduces SUMOylation (Gonzales-Santamaria *et al*. 2012; Bawa-Khalfe *et al*. 2016). An important difference is that all of these studies examined tagged, recombinant SUMO2 or SUMO 2/3 except Bawa-Khalfe *et al*. (2016), while my work has tested endogenous SUMO2/3. Testing SUMOylation of target proteins with endogenous SUMO is valuable due to the possibility of confounding issues of increased SUMOylation when tagged recombinant SUMO is used (Eifler and Vertegaal, 2015).

I next examined how PTEN can influence AMPARs in neurons, again with the overall aim of exploring the relevance of PTEN to synaptic transmission dysregulation in Alzheimer's disease (Chang *et al*. 2006). My results suggest that PTEN positively regulates surface GluA2. My work so far did not reveal a clear mechanism behind this but it may be through PTEN's phosphatase action against CaMK2, or through its effects on PIP_3 sensing proteins such as SNX27 or PHLDB2, which will be outlined in this chapter (Xie *et al*. 2019; Arendt *et al*. 2010; Cai *et al*. 2001; Song and Huganir 2002; Qin *et al*. 2005). Interestingly, PTEN-3KR was also defective in Akt pathway regulation, supporting evidence that SUMOylation or ubiquitination may also play a role in controlling PTEN phosphatase activity (Huang *et al*. 2012).

I then tested how PTEN can influence retromer mediated trafficking, a process known to be disrupted in AD, which can also regulate GluA2 trafficking (Tian *et al*. 2015; Bhalla *et al*. 2012; Vagnozzi and Praticò, 2019; Ansell-Schultz *et al*. 2018). I created HeLa cell lines with modulations of PTEN to examine PTEN-mediated regulation of retromer cargo trafficking, and how PTEN effects retromer proteins. I discovered that PTEN-WT overexpression can upregulate SNX27 and VPS26 protein levels and PTEN-3KR expression can downregulate surface GluT1. This builds on reports that PTEN can influence retromer-mediated trafficking (Shinde and Maddika, 2017). The differential role of PTEN-3KR from PTEN-WT in both of these experiments suggests a potential role for SUMOylation, but more work is needed to

clarify this. I will now outline how I have fulfilled the aims outlined at the start of this thesis, then discuss my main findings.

1) To create and test tools to study PTEN SUMOylation/ ubiquitination

While I aimed to make a "non-SUMOylatable" PTEN mutant, PTEN-3KR has been a useful tool to examine enhanced SUMOylation (and ubiquitination). Creation of GFP-WT and GFP-PTEN-3KR has enabled me to detect endogenous SUMOylation and ubiquitination, which avoids potential confounding effects of overexpression.

2) To characterise these tools and examine how to changes to PTEN SUMOylation regulate its function

I have characterised aspects of PTEN-3KR function and properties including its ability to regulate phospho-Akt levels, dimerization and degradation. Notably, PTEN-3KR is defective in phospho-Akt regulation.

3) To test cross-regulation of SUMO and Ubiquitin on PTEN

My interpretation of my data leads me to tentatively suggest that SUMO can outcompete ubiquitin for modification on PTEN; overexpression of SUMO2 reduced ubiquitination of PTEN (although this result was just under the threshold for statistical significance (P=0.0541)). Although it is noted that this may be an artefact of overexpression, however, my results are in line with Wang *et al*. (2014) who report SUMOylation of PTEN can limit ubiquitination. My preliminary result using *in vitro* treatment with the de-SUMOylating enzyme SENP1 suggests that removal of SUMO with SENP1 treatment does not influence PTEN ubiquitination, suggesting ubiquitin is not forming chains on SUMO on PTEN.

4) To test the role of PTEN and PTEN SUMOylation/ ubiquitination in AMPAR trafficking

By examining both PTEN KD and overexpression, I have revealed a bidirectional effect of PTEN in regulating surface GluA2 levels. While the effect of PTEN KD in reducing surface GluA2 was not robust, possibly due to variability between batches of neurons, taken together they are suggestive of a role for PTEN in AMPAR trafficking. However, by comparing PTEN-WT and PTEN-3KR on GluA2 surface expression in surface biotinylation experiments, I observed no significant difference, suggesting that the increased SUMOylation status of PTEN does not play a role in this process.

5) To examine how PTEN affects retromer in terms of its trafficking ability and regulation of its components, and if SUMOylation/ ubiquitination are involved

My experiments revealed that WT, but not PTEN-3KR overexpression can upregulate SNX27 and VPS26 levels in HeLa cells. PTEN-3KR bound SNX27 significantly less than WT, and also reduced surface expression of the glucose transporter GluT1, which was surprising given that PTEN can sequester SNX27 and interrupt GluT1 forward trafficking (Shinde and Maddika, 2017). In contrast, PTEN KD or WT overexpression had no significant effect on surface GluT1, in contrast to previous work (Shinde and Maddika, 2017). This points to a possible role of PTEN SUMOylation in regulating GluT1 expression, but more experiments are needed to clarify whether it is SUMOylation or another quality of PTEN-3KR that is responsible for this effect. These experiments also suggest that the role of PTEN in GluT1 trafficking is more complex than purely through SNX27 sequestration.

7.2 Discussion of the Main Findings

7.2.1 PTEN-3KR is More SUMOylated than WT

I used PTEN-3KR to examine SUMOylation, which according to the literature would be non-SUMOylatable due to mutation of three lysines which are reported SUMO sites (Huang *et al*. 2012; Gonzalez-Santamaria *et al*. 2012; Bassi *et al*. 2013). Unexpectedly, in my experiments, PTEN-3KR is significantly more SUMOylated by endogenous SUMO2/3 than WT, and preliminary results suggest it may also be more SUMOylated by SUMO1. However, conversely, when I tested tagged, recombinant SUMO2, PTEN-WT was significantly more SUMOylated than PTEN-3KR, which is congruent with the literature showing single or double mutations of the three SUMO sites reduce PTEN SUMOylation by overexpressed SUMO. My experiments therefore suggest that tagged, recombinant SUMO may behave differently to endogenous SUMO. Eifler and Vertegaal (2015) point out that expression of exogenous SUMO can lead to increased SUMOylation of target proteins, and artefacts can occur through overexpression. According to this, the reduced SUMOylation of PTEN-3KR (and possibly other PTEN mutants in the literature) seen with tagged, recombinant SUMO are possibly an artefact, and assessing SUMOylation by endogenous SUMO as in Figure 4.3.4 may be more reliable.

Overexpression of SUMO can lead to artefacts in target protein SUMOylation if not controlled (Eifler and Vertegaal 2015). It is possible that WT is more SUMOylated in recombinant SUMO conditions, where SUMO is potentially being ‘pushed’ onto non-physiologically relevant sites, simply because it has more available lysines to modify. Therefore, overexpression of SUMO could force more SUMO onto available sites on PTEN-WT, which

are mutated and unavailable for SUMO to attach to on PTEN-3KR. Eifler and Vertegaal (2015) suggest that target protein identification should also be confirmed by examining endogenous SUMO after exogenous SUMO is used; my experiments in this thesis and Bawa-Khalfe *et al*. (2016) are the only current examples of this strategy for PTEN. This is important considering many of the conclusions drawn about the role of PTEN SUMOylation are based on the premise that the previously used lysine mutants are less SUMOylated, such as work on phosphatase activity (Huang *et al*. 2012).

7.2.2 Possible mechanistic explanations for how PTEN-3KR is more SUMOylated than WT

As outlined above, there is currently no clear explanation for how PTEN-3KR would have enhanced endogenous SUMOylation compared to WT. Possible reasons and approaches to resolve this issue include:

- **A change of structure or conformation of PTEN-3KR leading to more exposed SUMOylatable lysines.** This could be tested by the immunoprecipitation experiment suggested in 4.6., whereby tagged mutants of the PTEN C-tail and the rest of WT or PTEN-3KR can be immunoprecipitated (Odriozola *et al*. 2007; Rahdar *et al*. 2009). If PTEN-3KR was able to immunoprecipitate more C-tail fragment than WT, this would imply it exists more in the “closed” conformation, due to enhanced ability to form intramolecular interactions between the C-tail and PTEN-3KR. PTEN-3KR and PTEN-WT appear to dimerise with PTEN-WT to the same extent, suggesting there is not likely to be major changes to structure/conformation.

- **Removal of lysines in PTEN-3KR that are modified by SUMO or other PTM’s, which may be blocking SUMOylation at other sites; Huang et al. (2012) suggest concomitant SUMOylation at K254 and K266 is impossible due to the large size of SUMO.** Therefore, removal of these lysines and their modifications may allow more space for modifications at other potential SUMO sites which could increase total PTEN SUMOylation. Testing the level of endogenous SUMOylation of individual mutants, using the same experimental procedure used here, would help to narrow down whether one, or a combination of mutations in PTEN-3KR are causing the increase.

- **Removal of lysines mutated in PTEN-3KR may increase the interaction of SUMO with an adjacent SIM site.** Increased interaction between SIMs and SUMO can bring

SUMO closer to other potential SUMO sites which it could modify, increasing SUMOylation (Wang and Dasso 2009). Bawa-Khalfe *et al*. (2016) have identified two potential SIM sites in PTEN: amino acids 98-101 and 317-320. Removal of lysines in PTEN-3KR may potentially enhance the availability of these SIM sites, increasing SUMOylation. A co-immunoprecipitation under native conditions would help to detect non-covalent binding of SUMO to SIM sites on PTEN. This could be carried out with non-conjugatable SUMO, which lacks the C-terminal diglycine motif necessary for SUMOylation, so could only interact with PTEN non-covalently (Kantamneni *et al*. 2011; Saether *et al*. 2011). Additionally, known SIM sites could be mutated to see if this affects SUMOylation of PTEN-3KR.

7.2.3 PTEN, SUMO and Membrane Localisation

Membrane localisation of PTEN is critical for its role in P-Akt regulation (Vazquez *et al*. 2000). Huang *et al*. (2012) suggest that membrane localisation is the link between SUMOylation and PTEN phosphatase activity, and postulate that SUMOylation at K266 on PTEN facilitates electrostatic interactions between PTEN, SUMO and the membrane, driving phosphatase activity. Furthermore, Gonzales Santamaria *et al*. (2012) suggest SUMO modification of PTEN forces it into the “open” conformation by blocking interactions between the C-tail and C2 domain, facilitating membrane localisation and phosphatase activity. Consequently, the general consensus in the literature is that SUMOylation facilitates phosphatase activity of PTEN.

In contrast, my results demonstrate that PTEN-3KR, exhibits significantly less ability to supress P-Akt than PTEN-WT, yet is more SUMOylated (Figures 5.3.3. and 4.3.4), these data are incongruent with the notion that SUMOylation increases PTEN “open” conformation and consequent membrane binding proposed by Gonzales-Santamaria (2012). This suggests that SUMO may not facilitate phosphatase activity through membrane binding, and is more in line with Shenoy *et al*. (2012), who argue SUMOylation of PTEN would be more likely to reduce membrane localisation.

I note that it is not clear where on PTEN-3KR SUMOylation is occurring, and Gonzales-Santamaria *et al*. (2012) do not suggest which sites of SUMOylation keep PTEN “open”. It may be that SUMOylation of specific sites such as K266 facilitates the open conformation and membrane binding (Huang *et al*. 2012), but sites modified on PTEN-3KR have an inhibitory effect on membrane binding. Alternatively, it may be that additional SUMO on PTEN-3KR is not influencing conformation or membrane binding, but the additional SUMO

reduces phosphatase activity via another route, such as by obscuring the PIP_3 binding site. Another possibility is that since SUMOylation must be tightly regulated as it can have a strong influence on the function of target proteins (Schorova and Martin 2016), extremes of both hypo- and hyper- SUMOylation of PTEN may cause its dysregulation and lead to enhanced Akt phosphorylation.

Shenoy *et al*. (2012) dispute the model proposed by Huang *et al*. They suggest that SUMO has the potential to be disruptive to membrane association according to molecular dynamics (MD) simulations, which can be used to simulate behaviour of proteins over time (Hollingsworth and Dror 2018). Considering that I have found mutations used by Huang *et al*. (2012) may actually increase endogenous PTEN SUMOylation, Shenoy's conclusion seems more consistent with my findings. There are several, non-mutually exclusive possibilities which could explain the discrepancy between my results and those of Huang (2012) and Gonzales-Santamaria *et al*. (2012):

1) Huang's K266R mutant, like PTEN-3KR, is in fact more SUMOylated than PTEN-WT in the absence of overexpressed SUMO, and this actually inhibits PTEN phosphatase activity.
2) PTEN's SUMOylation level needs to be tightly controlled (Schorova and Martin, 2016): both inhibition or enhancement of SUMOylation blocks P-Akt regulation.
3) Discrepancies are a result of differences in methodology or cell type
4) Additional SUMO on PTEN-3KR may not be directly involved in the reduction of PTEN phosphatase activity; other aspects of PTEN-3KR such as a change in conformation may be responsible.
5) Effects of SUMOylation may be site specific; SUMOylation at K266 may aid membrane localisation, while SUMOylation at other sites may hinder it.

In support of possibility 1), Shenoy *et al*. (2012) have suggested that Huang's model whereby SUMO facilitates membrane binding electrostatically is unlikely, based on a few observations. Firstly, they point out that according to the model, K266 would be likely to be frequently mutated in cancer, but this has not been shown (Liu *et al*. 2019; Shenoy *et al*. 2012). Huang *et al*. (2012) suggest that SUMO1 essentially sits at the side of PTEN and touches the membrane, and lysines K237, K263 and K31 in PTEN also touch the membrane. Shenoy *et al*. (2012) also state that in order for SUMO-PTEN to associate with the membrane and for SUMO1 to facilitate this by touching the membrane, PTEN would have to be in a different orientation to the one suggested by Lee *et al*. (1999), who made predictions based on the crystal structure. Shenoy *et al*. (2012) carried out neutron reflectometry

to further test the original model, a technique which involves measuring reflected scattered neutrons that have been directed at a surface, which can be used to measure membrane structure (Shenoy *et al*. 2012; STFC (ISIS Neutron and Muon Source)). Their results are congruent with the orientation suggested by the original crystal structure experiments by Lee *et al*. (1999), and support a model whereby PTEN does not bind the membrane in the orientation suggested by Huang *et al*. (2012). Overall, my results support Shenoy's conclusions, as they suggest SUMOylation of PTEN is more likely to have an inhibitory effect on membrane localisation, which would reduce phosphatase activity (Rahdar *et al*. 2009; Vazquez *et al*.
2006). This is line with PTEN-3KR, which has enhanced SUMOylation, and also displays reduced activity against P-Akt. Alternatively, it may be that K266 SUMOylation positively regulates membrane association and activity, but enhanced SUMOylation at other sites has the opposite effect.

In summary, it seems possible that SUMO could hinder membrane association and activity against P-Akt, and differences seen in the SUMOylation level of mutants between this and previous work are most likely due to discrepancies between endogenous and tagged, recombinant SUMO. However, it cannot be ruled out that effects of SUMOylation on membrane localisation and activity are site specific. It may be that SUMO at K266 drives membrane association and Akt regulation (Huang *et al*. 2012), but additional SUMO at other sites has the opposite effect.

7.2.4 PTEN-3KR Ubiquitination

As well as increased levels of SUMOylation, PTEN-3KR was also more ubiquitinated. It is possible that effects seen when comparing PTEN-3KR to PTEN-WT could also be due to this additional ubiquitin, rather than SUMO (or both). SUMO-ubiquitin hybrid chains can act as a signal at DNA damage sites to promote DNA repair (Guzzo *et al*. 2012). However, while interpreted with caution due to lack of repeats, *in vitro* treatment with SENP1 enzyme removed SUMO2/3 from PTEN immunoprecipitated from HEK293T cells, but did not alter ubiquitination of PTEN, suggesting ubiquitin is not attaching to PTEN via SUMO.

Ubiquitination can often act as a signal for degradation, as well as induction of signalling cascades (Kawadler and Yang, 2006; Tatham *et al*. 2008). PTEN K289 polyubiquitination is associated with proteasome-mediated degradation (Wang *et al*. 2003), however I found that PTEN-WT and PTEN-3KR were degraded to a similar extent after 24-hour cycloheximide treatment (Figure 5.3.1.1.). This suggests that the additional ubiquitin on PTEN-3KR is likely not the degradation-associated Lys48-linked chain (Kawadler and Yang, 2006; Haglund *et al.*

2003; Hershko and Ciechanover, 1998; Komander, 2009; Hicke *et al.* 1996). Blotting with

chain- specific ubiquitin antibodies would help to validate this, and establish which other ubiquitin chain linkage is preferentially modifying PTEN-3KR. For example, it could be that the additional ubiquitin is Lys63 linked, which can induce signal cascade activation (Kawadler and Yang, 2006).

7.2.5 PTEN and Retromer Component Upregulation

While few studies have examined the role of PTEN on retromer, there is evidence that PTEN can inhibit retromer formation through sequestration of SNX27 (Shinde and Maddika, 2017). I wanted to further examine this, due to evidence that retromer is critically involved in plasticity, and may be dysregulated in AD (Temkin *et al*. 2017; Tian *et al*. 2015; Munsie *et al*. 2014; Li *et al*. 2019; Choy *et al*. 2014; Wang *et al*. 2012). Furthermore, as GluA2 is a retromer cargo (Tian *et al*. 2015), I wondered if PTEN was positively influencing surface GluA2 via retromer. I therefore created HeLa cell lines with PTEN modulations as a model system to examine the effects of PTEN on retromer proteins and trafficking of model cargoes.

PTEN-WT overexpression increased protein levels of the retromer components VPS26 and SNX27, an effect which has not previously been reported. A potential explanation for this comes from recent work on transcription factor TFEB, a transcription factor which upregulates transcription of many genes in the lysosomal pathway (Sardiello *et al*. 2009), and can upregulate expression of the retromer components SNX27, VPS26 and VPS35 (Curnock *et al*. 2019). PTEN is a target of TFEB, and can also indirectly cause its activation through the proposed feedback loop whereby PTEN can block inhibitory TFEB phosphorylation by Akt (Palmieri *et al*. 2017; Palmieri, Pal, and Sardiello 2017; Polito *et al*. 2014; Settembre *et al*. 2012). To more vigorously validate the role of TFEB in the PTEN-mediated upregulation of VPS26 and SNX27, expression of these proteins should be measured after TFEB removal by shRNA knockdown in control and PTEN overexpression conditions. RT-PCR could also be used to examine if SNX27 and VPS26 are being regulated at the transcriptional level under these conditions.

Unlike PTEN-WT, PTEN-3KR did not upregulate SNX27 and VPS26 levels (Figure 6.3.3.1. and Schematic 6.4.1.). As PTEN-3KR has reduced activity against P-Akt, this suggests the ability of PTEN to upregulate SNX27 and VPS26 may be dependent on its phosphatase activity. This further suggests the involvement of TFEB, as PTEN-3KR is less able to supress P-Akt, which is reported to have an inhibitory effect on TFEB (Polito *et al*. 2014; Palmieri *et al*. 2017). It also suggests a possible role for SUMOylation in PTEN's influence over these proteins, but these experiments should be repeated under conditions which specifically

modulate SUMOylation, for example SENP overexpression conditions, to confirm the involvement of SUMO.

TFEB is relevant in AD pathology as its expression can also increase the uptake and lysosomal degradation of Aβ42 in primary astrocytes in APP/PS1 mice (Xiao *et al*. 2014; Martini-Stoica *et al*. 2016). Furthermore, TFEB activation via PTEN can clear Tau in AD mouse model rTg4510 brains; lipid phosphatase activity of PTEN is critical in Tau clearance (Polito *et al*. 2014). TFEB activation can also reduce neurodegeneration in the form of aberrant hippocampal volume and neuroinflammation, and can increase neuronal survival (Polito *et al*. 2014). TFEB upregulation can increase 1A/1B-light chain 3 (LC3-2)-positive autophagosome clearance, which is a proposed mechanism of p-Tau clearance (Poltito *et al*. 2014). Retromer is also implicated in protein aggregate clearance via autophagy (Cui *et al*. 2019; Carosi *et al*. 2020).

Therefore, it is plausible that PTEN, through TFEB-mediated control of retromer, could alter the autophagy-lysosomal pathway (Polito *et al*. 2014; Cui *et al*. 2019). More work in therefore needed to clarify whether the role of PTEN in TFEB-mediated regulation of autophagic proteins shown in these studies could involve retromer. A starting point for this could be examining whether the ability of PTEN-WT overexpression to upregulate VPS26 in HeLa cells is also observed in neurons, and whether PTEN-mediated aggregate clearance via TFEB also involves VPS26 (Polito *et al*. 2014).

7.2.6 PTEN and SNX27 Binding

Another potential explanation for the increase in SNX27 when PTEN is overexpressed is that PTEN can sequester SNX27 (Shinde and Maddika, 2017). Thus, SNX27 could be upregulated to compensate for this. As PTEN-3KR binds SNX27 significantly less than PTEN-WT, this could explain why SNX27 is not upregulated by PTEN-3KR expression. While PTEN overexpression did not increase SNX27 in neurons, it should be examined whether PTEN attenuation is able to change the localisation of PIP_3-sensing SNX27 through modulation of PIP_3 levels (Cullen 2008; Ghai *et al*. 2015; Cai *et al*. 2011). This may have an effect on APP trafficking and Aβ generation as SNX27 interacts with γ-secretase complex subunit Presenilin 1 (PS1), disrupting stability of the γ-secretase complex and limiting Aβ generation (Wang *et al*. 2014).

Furthermore, as the PTEN PDZ binding motif can bind SNX27 via its PDZ domain and sequester it (Shinde and Madikka, 2017), and SNX27 PDZ domain interacts with PS1, its possible that PTEN could interrupt SNX27-PS1 interactions. It would be interesting to

examine whether the SNX27 sequestration by PTEN seen by Shinde and Maddika (2017) can disrupt the role of SNX27 in limiting stability of PS1, which could enhance Aβ generation (Wang *et al*. 2014). As PTEN-3KR was less able to bind SNX27, its possible that SUMOylation and/or ubiquitination could play a role in this.

The finding that PTEN-3KR bound SNX27 less than WT, may have implications for various neuronal receptors trafficked by SNX27 such as NRC2, given that PTEN can sequester SNX27 and block forward trafficking of other cell surface proteins such as GluT1 (Cai *et al*. 2011; Shinde and Maddika, 2017). Conducting surface biotinylation experiments to measure surface NRC2 in PTEN KD and overexpression conditions may provide more mechanistic insight into how PTEN phosphatase activity plays a critical role in NMDAR-mediated plasticity (Jurado *et al*. 2010). More work is also needed to establish whether PTEN can sequester SNX27 in neurons and block retromer formation (Shinde and Maddika *et al*. 2017), and if differential binding between WT and PTEN-3KR exists in neurons and influences trafficking.

7.2.7 PTEN and Retromer - GluT1 and ASCT2 Trafficking

Due to previous work showing that PTEN can bind SNX27 and limit forward GluT1 trafficking, I explored the role of PTEN SUMOylation in this by testing PTEN-3KR alongside PTEN-WT and PTEN KD conditions in surface biotinylation assays. Unexpectedly, PTEN-3KR, which bound SNX27 less than WT, also significantly reduced GluT1 on the surface, while WT and KD had no significant effect. Thus, since only PTEN-3KR had an effect, it seems that PTEN-3KR had a gain of function in reducing surface GluT1.

I do not currently have a clear explanation for why PTEN-3KR would limit GluT1 surface expression more than PTEN-WT, indeed this is even more surprising given the lack of activity of PTEN-3KR against P-Akt, and the observations that PI3K pathway inhibition can reduce surface GluT1, and Akt activation is associated with increased surface GluT1 (Wieman, Wofford, and Rathmell 2007). To establish if the lack of P-Akt suppression from PTEN-3KR is relevant to its effect of surface GluT1, PTEN-3KR could be compared with a catalytically dead mutant. If this mutant also reduces surface GluT1, it is likely that the effect seen for PTEN-3KR is due to a lack of activity against P-Akt. This could be tested further with Akt inhibitors, or by PIP_3 synthesis inhibition using a PI3K inhibitor in PTEN-3KR and WT conditions, to examine whether it is an increase of PIP_3 or Akt activation that is causing the effect. The experiment could also be repeated with the addition of SENP and a DUB, in order to remove the additional SUMO/ ubiquitin from PTEN and examine whether this is influencing GluT1. ASCT2 surface levels were not significantly affected by PTEN KD, WT or PTEN-3KR

expression, suggesting PTEN-3KR does not influence all retromer cargos in HeLa cells (Scalise *et al*. 2018).

7.2.8 PTEN and GluA2 Trafficking – PIP_3 and AMPAR Regulation

While the initial finding that PTEN KD reduces surface expression of GluA2 in neurons was not robust, taken with the finding that PTEN over expression increases surface GluA2, these experiments suggests that PTEN can positively regulate surface GluA2 levels. This was slightly unexpected given that PTEN inhibition has been linked to increased surface AMPAR expression (Liu *et al*. 2013; Moult *et al*. 2010), although GluA2 levels after PTEN KD have not previously been examined.

Despite a lack of clear link between PTEN and GluA2 surface levels and the fact that knowledge of PTEN function in neurons in limited, a possible explanation for PTEN's role in GluA2 trafficking is though PIP_3 regulation. Phosphoinositide dephosphorylation in regulation of the membrane plays an important role in AMPAR trafficking (Parkinson and Hanley 2018); Arendt *et al*. (2010) report that PIP_3 depletion causes reduction of AMPARs at the synaptic membrane, and depression of AMPAR-mediated transmission. Although these mechanisms are not well characterised, PIP_3 may regulate AMPAR clustering at the membrane through modulation of the PSD-95 synaptic complex, specifically by enabling PSD-95 accumulation in spines (Arendt *et al.* 2010; Elias *et al.* 2006; Bats *et al.* 2007). Therefore PTEN, through lipid phosphatase activity against PIP_3, may influence the ability of PSD-95 to anchor AMPARs at synapses, reducing their clustering at the membrane (Arendt *et al.* 2010; Maehama and Dixon, 1999; Bats *et al.* 2007).

PIP_3 may also play a role in AMPAR phosphorylation; phosphorylation of GluA1 at S831 is driven by signalling through the Ras–Pi3K–Akt pathway, which facilitates GluA1 insertion into synapses (Qin *et al*. 2005). Qin *et al*. (2005) suggest that rather than phosphorylating GluA1 directly, Akt activates calcium/calmodulin-dependent protein kinase 2 (CaMKII) which could phosphorylate GluA1 (Song and Huganir, 2002). This implicates the PI3K/Akt pathway in AMPAR trafficking; it's possible that this pathway could be influenced by PTEN. Indeed, in hippocampal neurons, reducing PTEN levels enhances Akt phosphorylation (Ning *et al*. 2004), so potentially PTEN could influence AMPAR insertion through counteracting PI3K signalling and reducing AMPAR phosphorylation, which could limit AMPAR insertion into synapses (Qin *et al*. 2005).

Although PTEN-mediated PI3K/Akt downregulation would likely reduce GluA1 phosphorylation and subsequent synaptic insertion according to some of the literature, other

work has shown PTEN isoform PTENα can dephosphorylate CaMKII at T305/306, facilitating its activation (Wang *et al*. 2017). CaMKII also is necessary for GluA2 release from the endoplasmic reticulum (ER), which influences GluA2 synaptic delivery and surface expression (Lu, Khatri, and Ziff 2014). PTENα KO mice display CaMKII-mediated LTP and spatial learning deficits (Lu, Khatri, and Ziff 2014). CaMKII can also phosphorylate Stargazin, stabilizing it and enabling its binding to PSD-95, which can trap AMPARs at the synapse (Bats, Groc, and Choquet 2007; Opazo *et al*. 2010).

With this in mind, it is possible that the increase in surface GluA2 levels seen under PTEN overexpression conditions is due to increased CaMKII activation, which could enhance GluA2 ER release and forward trafficking. Imaging of GluA2 with an ER marker under PTEN KD and overexpression conditions would reveal whether PTEN is able to regulate GluA2 ER release. If this was blocked by KD/inhibition of CaMKII, this would suggest PTEN is increasing forward trafficking of GluA2 via enhancing CaMKII activation (Lu *et al*. 2014). Future experiments could also involve examining the phosphorylation status of GluA1 and GluA2 in PTEN KD and overexpression conditions, to establish whether PTEN can regulate phosphorylation-mediated AMPAR insertion (Qin *et al*. 2005). It has not been reported that PTEN can directly dephosphorylate AMPARs, although it is also possible that PTEN may influence AMPAR phosphorylation indirectly for example via the Ras–Pi3K–Akt pathway (Qin *et al*. 2005).

Similar to what has been presented here for AMPARs, PTEN levels have a positive effect on NMDAR surface expression (Ning *et al*. 2004). PTEN KD decreases NMDAR surface expression and currents, which is attributed to loss of PTEN phosphatase activity (Ning *et al*. 2004). Surface NR1 levels were increased in G129E mutant PTEN (lipid-phosphatase inactive) expressing neurons, but reduced in neurons expressing C124A mutant (lipid and protein-phosphatase inactive) (Ning *et al*. 2004; Myers *et al*. 1998; Weng *et al*. 2001), suggesting both lipid and protein phosphatase activities of PTEN (outlined in 1.2.2 and 1.2.3) can have different effects of surface NMDAR expression. It also suggests that downregulation of PTEN protein phosphatase activity can reduce surface levels of NMDARs (Ning *et al*. 2004). The mechanism by which surface NMDARs are regulated by PTEN protein phosphatase activity is not clear, but it would be useful to examine whether the effect of increased surface AMPARs under PTEN overexpression conditions is also related to PTEN protein phosphatase activity. PTEN can associate with NMDARs, but it has not been examined whether it can associate with AMPARs, or whether it can dephosphorylate them.

Comparing PTEN-WT with a phospho-inactive mutant in future experiments may elucidate whether the phosphatase activity of PTEN is involved in surface GluA2 regulation. It would be useful to compare G129E mutant PTEN (lipid-phosphatase inactive) and C124A mutant (lipid and protein-phosphatase inactive) (Myers *et al*. 1998; Weng *et al*. 2001), to examine which aspect of phosphatase activity may influence AMPARs.

7.2.9 PTEN and GluA2 Trafficking – PHLDB2

Pleckstrin Homology Like Domain Family B Member 2 (PHLDB2), has a PIP_3-sensing Pleckstrin Homology (PH) domain, and can also interact with GluA1, GluA2 and PSD-95 (Levi *et al*. 1993; Xie *et al*. 2019). Recently it has been shown that PHLDB2, through sensing PIP_3, can regulate plasticity and AMPAR and NMDAR localisation (Xie *et al.* 2019). PHLDB2 localises in spines in neurons, and is highly sensitive to PIP_3 which plays a fundamental role in its localisation (Xie *et al.* 2019). Its localisation is also influence by Brain Derived Neurotrophic Factor (BDNF), which causes PHLDB2 translocation to spines (Xie *et al.* 2019). BDNF is an important regulator of LTP, by activating Tropomyosin Receptor Kinase B (Trkb); Trkb signalling can regulate spine growth and activity-Dependent remodelling (Xie *et al*. 2019; Korte *et al*. 1995; Lai *et al*. 2012; De Vincenti *et al*. 2019).

PHLDB2 interacts with and regulates PSD-95 turnover and localisation, and is critical in LTP. GluA2 was reduced at the plasma membrane in hippocampal neurons from PHLDB2 KO mice, shown in imaging experiments. Expression of GFP- PHLDB2 significantly restored this deficit. Rescue with a PHLDB2 mutant lacking the PH domain was not able to rescue plasma membrane GluA2, suggesting PHLDB2-PIP_3 interactions are important in regulating surface GluA2 (Xie *et al*. 2019). Furthermore, LY294002 treatment, used to inhibit PIP_3 synthesis, decreased GluA2 at the membrane in WT, but not PHLDB2 KO mice. It was suggested that PHLDB2 regulates AMPAR surface expression downstream of PIP_3 signalling (Xie *et al.* 2019). In addition, NR1 was also reduced in PHLDB2 KO mice, which also exhibited a lack of CA1 LTP and deficits in reference memory. Xie *et al*. (2019) postulate that through PIP_3 sensing, PHLDB2 helps PSD-95 to accumulate in spine heads, which regulates NMDAR trafficking to the synapse, and stabilises NMDARs at the membrane. It was concluded that PHLDB2 is indispensable in plasticity, through its ability to sense membrane phosphoinositides (Xie *et al.* 2019). Considering this study, PTEN-mediated changes in PIP_3 levels could influence localisation of PHLDB2, which could have downstream effects on surface AMPARs and NMDARs. To test this, PHLDB2 could be imaged in neurons in PTEN KD and WT overexpression conditions to establish whether its localisation changes. The involvement of PIP_3 in these conditions could be confirmed with use of PI3K inhibitor

LY294002 as used by Xie *et al*. (2019). PHLDB2 could then be knocked out; if this ablates the effect of PTEN KD and WT overexpression, this would suggest a PTEN-mediated role of PHLDB2 in regulating surface GluA2 expression, and that PTEN may be influencing PHLDB2 localisation via regulation of PIP_3 levels.

7.2.10 PTEN and GluA2 Trafficking – SNX27

SNX27 is involved in trafficking of various neuronal cell-surface proteins including AMPARs, NMDARs, Neuroligin-2 and Transferrin receptor (Temkin *et al*. 2011; Cai *et al*. 2011; Halff *et al*. 2019; Hussain *et al*. 2014; Wang *et al*. 2013). In neurons, SNX27 is also localised to spines and the postsynapse (Loo *et al*. 2014). SNX27 overexpression can upregulate surface GluA1, GluA2 and NMDAR subunit NR1, and SNX27 KD has the opposite effect (Wang *et al*. 2013). SNX27 overexpression can also increase AMPAR exocytosis during LTP, and SNX27 KD can reduce surface AMPAR delivery, blocking LTP in mouse hippocampal slices (Loo *et al*. 2014). Although SNX27 is generally associated with endosome to membrane trafficking of membrane proteins (Shinde and Maddika, 2017; Hussain *et al*. 2014), KD of SNX27 has also been shown to increase surface levels of over 20 other cell surface proteins including Sodium- and Chloride-Dependent Creatine Transporter 1 and Ephrin B1 (Steinberg *et al*. 2013). It is not clear exactly how SNX27 has this effect, but it has been found to regulate membrane endocytosis of NMDAR subunit NR2C (Cai *et al*. 2011). SNX27 physically interacts with NRC2 via its PDZ domain and cortical neurons from SNX27 knockout mice have increased surface NRC2 levels shown in imaging experiments, indicating reduced NRC2 endocytosis (Cai *et al*. 2011). SNX27 is also reported to facilitate Multidrug Resistance-associated Protein 4 (MRP4) endocytosis, negatively regulating its availability at the surface (Hayashi *et al*. 2012). It is thought that PDZ-interactions with MRP4 near the plasma membrane facilitate this process (Hayashi *et al*. 2012). SNX27 therefore has a role in endocytosis, which may be separate to its role in endosomal sorting and recycling (Burd and Cullen 2014; Cai *et al*. 2011; Hayashi *et al*. 2012; Shinde and Maddika 2017).

Considering these studies, it is possible that in neurons, PTEN has influence over the role of SNX27 in reducing GluA2 endocytosis at the membrane. A role for SNX27 in AMPAR endocytosis has not yet been shown, but SNX27 is reported to regulate AMPAR exocytosis (Wang *et al*. 2013; Hussain *et al*. 2014). SNX27 levels did not change significantly under PTEN KD, WT KD-rescue or PTEN-3KR KD-rescue conditions in neurons, suggesting that PTEN is not having the same influence on SNX27 in neurons as in HeLa cells (Figures 6.3.4. and 6.3.3.), and suggesting PTEN is not upregulating surface GluA2 through increasing SNX27 total levels. However, PTEN may influence the localisation of SNX27 at the post-

synapse or endosomes and reduce its ability to endocytose AMPARs via three potential mechanisms. Firstly, PTEN may influence SNX27 localisation and trafficking through modulation of PIP_3 levels, as PIP_3 can bind SNX27 at both at its Phox Homology (PX) domain and Four point one, Ezrin, Radixin, Moesin (FERM) domain, this can influence its localisation to early endosomes (Cullen 2008; Ghai *et al*. 2013; Ghai *et al*. 2015; Cai *et al*. 2011). SNX27 can is also enriched in spines and at the post-synapse (Loo *et al*. 2014). The likely decreased PIP_3 levels under PTEN overexpression conditions could therefore influence SNX27 localisation at the post synapse or/ and endosomes and reduce its ability to endocytose receptors, given that PIP_3 levels are involved in SNX27 targeting to dendrites, the synapse and endosomes (Cullen, 2008; Loo *et al*. 2014; Hussain *et al*. 2014; Cai *et al*. 2011; Ghai *et al*. 2015). It is not yet known whether the localisation of SNX27 at the post synapse is relevant to its ability to endocytose NRC2, but this would be interesting to test via imaging experiments (Cai *et al*. 2011; Loo *et al*. 2014).

Secondly, sequestration of SNX27 by PTEN could block direct interactions with AMPARs (Shinde and Maddika, 2017; Hussain *et al*. 2014). The PDZ ligands of NRC2 and MRP4 can bind SNX27 and this is suggested to underpin SNX27-mediated NRC2 and MRP4 endocytosis (Hayashi *et al*. 2012; Cai *et al*. 2011). It is possible that a similar mechanism could exist with AMPARs, which are also reported to bind the SNX27 PDZ domain (Hussain *et al*. 2014; Loo *et al*. 2014). SNX27 can also bind PTEN through its PDZ ligand, so PTEN could potentially block SNX27 binding with PDZ-containing proteins such as AMPARs, in the same way as it can block SNX27-VPS26 binding, which could compromise endocytosis/ trafficking of receptors (Hussain, *et al*. 2014; Shinde and Maddika, 2017). PTEN sequestration of SNX27 may also perturb its ability to bind the adaptor protein Leucine Rich Repeat and Fibronectin Type III Domain Containing 2 (LRFN2), which also associates with the SNX27 PDZ domain and can traffic AMPARs (McMillan *et al*. 2020).

Lastly, SNX27 sequestration can block retromer formation, a process known to influence cell-surface protein trafficking (Shinde and Maddika, 2017). Enhanced levels of PTEN could therefore reduce the ability of SNX27 to endocytose AMPARs, through blocking retromer formation, rather than through blocking direct SNX27-AMPAR interactions (Figure 7.2.2.1). It may be that PTEN is blocking SNX27-VPS26 association, and this reduces AMPAR endocytosis. The increase in VPS26 seen under PTEN overexpression conditions (Figure 6.3.3.) could be an attempt by the cell to compensate for this. Of note, VPS26 interaction enhances the affinity of SNX27 for binding with PDZ-motifs (Gallon *et al*. 2014).

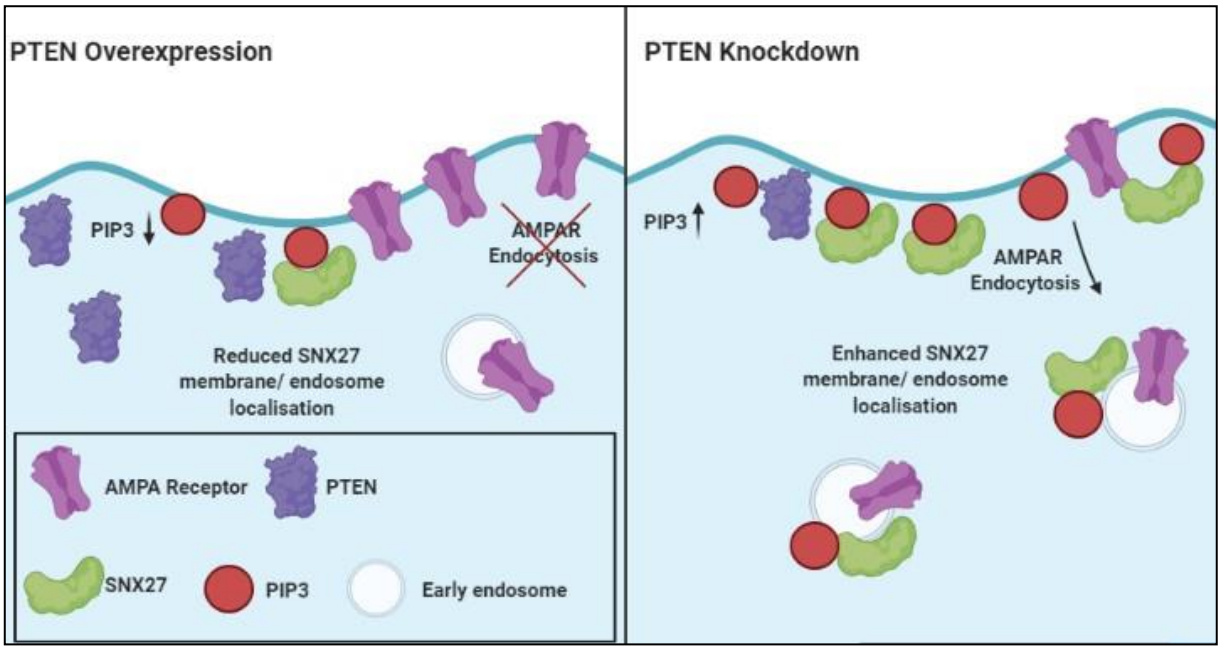


Figure 7.2.10.1. PTEN may influence SNX27-mediated AMPAR endocytosis via modulation of PIP_3 levels.

PTEN negatively regulates PIP_3 levels (Maehama and Dixon 1998). SNX27 is enriched at the postsynapse and early endosomes, however, changes in PTEN-mediated PIP_3 levels may alter the distribution of SNX27, due to the role of PIP_3 binding in its localisation (Cai *et al*. 2011; Wang *et al*. 2013; Loo *et al*. 2014). The likely PIP_3 level increase during PTEN KD could reroute SNX27 away from early endosomes, possibly reducing its ability to endocytose AMPARs. This is according to work showing that MRP4 and NRC2 endocytosis are regulated by SNX27 (Hayashi *et al*. 2012; Cai *et al*. 2011). This schematic is adapted from Sansal an Sellers (2004), and created in biorender with premade shapes.

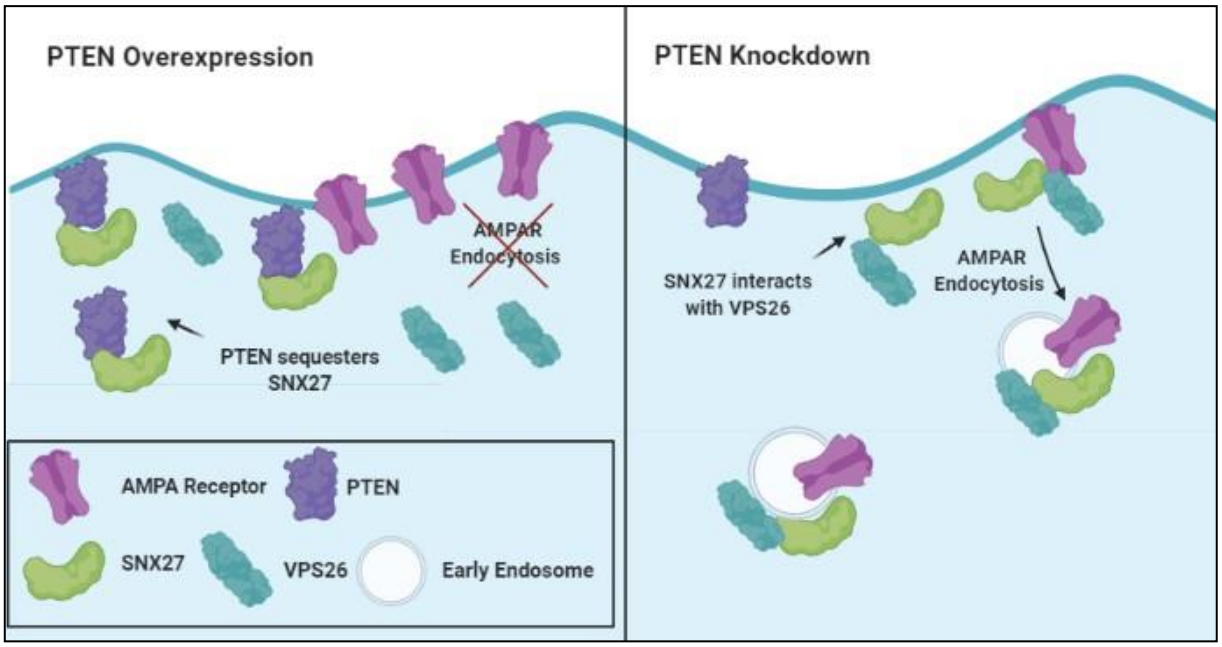


Figure 7.2.10.2. PTEN may influence SNX27-mediated AMPAR endocytosis via SNX27 sequestration.

Potentially enhanced SNX27 sequestration by PTEN in overexpression conditions may limit the availability of SNX27 at endosomes and the post-synapse (Ghai, 2015; Loo, *et al*. 2014; Shinde and Maddika, 2017). SNX27 depletion at these areas could reduce the ability of SNX27 to endocytose AMPARs, considering that SNX27 can endocytose NRC2 and MRP4 (Hayashi *et al*. 2012; Cai *et al*. 2011). This could occur via sequestration of the PDZ domain of SNX27 blocking direct SNX27- AMPAR interactions, or block interactions with adapter protein LRFN2, processes both involved in AMPAR trafficking (Loo *et al*. 2014; Hussain *et al*. 2014; McMillan *et al*. 2020). Alternatively, PTEN sequestration of SNX27 may block the ability of SNX27 to associate with VPS26, which may also disrupt trafficking of AMPARs due to evidence of the involvement of retromer in AMPAR trafficking (Shinde and Maddika, 2017; Temkin *et al*. 2017). During PTEN KD, there may be less SNX27 sequestration, and therefore more SNX27 available to endocytose AMPARs, leading to enhanced AMPAR endocytosis. Adapted from Sansal an Sellers (2004) and Shinde and Maddika (2017) and created in Biorender.com with premade shapes.

Knocking down SNX27 and repeating PTEN KD/ overexpression surface biotinylations experiments to see if this changes the effect on GluA2 levels would confirm a role of SNX27. Comparing co-localisation of SNX27 with PIP_3 and synaptic and endosomal markers between PTEN conditions would then begin to elucidate whether PTEN effects synaptic or endosomal SNX27 localisation. It would also be useful to immunoprecipitate PTEN in neurons and blot for SNX27, to see if these associate in neurons as they do in cell lines (Shinde and Maddika, 2017). Comparing surface GluA2 levels between WT and phospho-dead PTEN conditions, using LY294002 to inhibit PI3K and block PIP_3 synthesis (Qin *et al*. 2005), would help to confirm whether the phosphatase activity of PTEN is diving its positive

regulation of surface GluA2. It would also be interesting to see if PTEN can influence VPS26 or LRFN2 levels or activity in neurons and if this is relevant to GluA2 trafficking. This could be done by blotting and imaging of these proteins after expression of PTEN-WT or KD lentiviral constructs in neurons, to see if their expression or localisation changes. Additionally, VPS26 or LRFN2 could then be knocked down concomitantly with PTEN overexpression to see if this ablates the effect of PTEN on surface GluA2 levels.

The lack of difference between WT and PTEN-3KR on surface GluA2 suggests that enhanced SUMOylation is not involved in PTEN-mediated GluA2 regulation. However, it is noted that both constructs were greatly overexpressed, which could negate differences between conditions. Ideally, this experiment should be repeated at endogenous expression levels. Although PTEN-3KR had reduce activity against P-Akt compared to PTEN-WT, vast overexpression of both constructs may negate potential differences between them.

In summary, there are a variety of ways through which PTEN could regulate GluA2 trafficking, but PIP_3-sensing proteins such as PHLDB2 or SNX27 may be involved (Cai *et al*. 2011; Xie *et al*. 2019; Shinde and Maddika, 2017). Establishing whether PTEN phosphatase activity is involved will elucidate whether this mechanism is via PIP_3, or another mechanism such as sequestration through direct interactions (Shinde and Maddika, 2017). Taking these studies into account, there are a variety of routes by which PTEN, through modulation of PIP_3 levels, could influence AMPAR surface expression. PIP_3 plays an important role in NMDAR surface expression in LTP, and even under basal conditions can regulate surface AMPARs (Xie *et al*. 2019; Arendt *et al*. 2010). It also has the ability to regulate localisation of proteins involved in glutamate receptor trafficking including SNX27 and PHLDB2 (Xie *et al*. 2019; McMillan *et al*. 2020; Cai *et al*. 2011).

7.3 Conclusion

In this thesis, I have examined aspects of PTEN function relevant to AD including Akt pathway regulation, AMPAR trafficking and retromer regulation and trafficking (Kwak *et al*. 2010; Rickle *et al*. 2004; Chang *et al*. 2006; Vagnozzi and Pratico 2019; Tian *et al*. 2015; Mecozzi *et al*. 2014; Bhalla *et al*. 2012). In addition, I have examined the involvement of SUMOylation and ubiquitination in these processes by characterising the novel PTEN-3KR mutant. PTEN-3KR is surprisingly is more SUMOylated and ubiquitinated than PTEN-WT by endogenous SUMO2/3 and ubiquitin. This has highlighted the importance of testing endogenous SUMO when examining SUMOylation level of proteins as suggested by Eifler

and Vertegaal (2015), as this method is less susceptible to confounding issues resulting from overexpression or tags. The work in this thesis has also given insight into how enhanced SUMOylation and ubiquitination might regulate PTEN function in terms of dimerization and ability to regulate the Akt pathway, although more work is needed to confirm the involvement of SUMO or ubiquitin.

Experiments in this thesis have revealed a role for PTEN in positively regulating surface GluA2 levels in neurons; surface GluA2 after PTEN KD has not previously been examined. This is contrast to Liu *et al*. (2013), who found PTEN inhibition increases surface GluA2 in hippocampal neurons. However, my experiments involved PTEN KD for 6/7 days, while Liu *et al*. (2013) inhibited PTEN for 2 hours. The differences in effect may also be explained through the ability of PTEN to regulate PIP_3 levels at the membrane. Mechanistically, PTEN could influence PIP_3-sensing proteins involved in AMPAR trafficking such as SNX27 or PHLDB2 (Cai *et al*. 2011; Xie *et al*. 2019). Additionally, modulation of PIP_3 levels may influence AMPARs through regulation of PSD-95, which can stabilise AMPARs at the synapse (Arendt *et al*. 2010; Bats *et al*. 2007). Both WT and PTEN-3KR upregulate surface GluA2 levels, but only PTEN-3KR can downregulate surface GluT1, suggesting that these cell surface proteins are not regulated by PTEN via the same mechanism. As PTEN levels enhanced surface GluA2, GluA2 is not affected by SNX27 sequestration through PTEN in the same way as GluT1 (Shinde and Maddika, 2017). This could mean that either PTEN does not sequester SNX27 in neurons, or that potential sequestration could influence endocytosis rather than forward trafficking. PTEN-3KR did not have significantly different effects to PTEN-WT in GluA2 surface expression; while this may be due to the high level of overexpression of the PTEN constructs masking any subtle effects, this suggests enhanced SUMOylation of PTEN does not influence surface GluA2 levels.

In summary, PTEN-3KR did have a differential role to WT in various aspects of cellular regulation relevant to cell surface protein trafficking including modulation of retromer component levels in HeLa cells, binding to SNX27, Akt pathway regulation, and surface GluT1 expression (Shinde and Maddika, 2017; Steinberg *et al*. 2013; Morani *et al*. 2014). While more research is needed to confirm the involvement of PTEN SUMO in effects of PTEN-3KR, this work is suggestive of a role for enhanced SUMOylation in influencing these processes. The addition of SENP1 in repeats of my experiments, either through recombinant expression in cells or treatment with the purified protein, would help elucidate this further. If the SENP1 treatment had no effect on the outcomes of the experiment(s), this would suggest that it is not enhanced SUMOylation that changes the activity of PTEN-3KR, and experiments could be carried out to establish the mechanism by which PTEN-3KR exhibits

its effect. This could include the addition of a DUB to examine the involvement of ubiquitination.

Nonetheless, the data presented here provide further insight into the regulation of PTEN by SUMOylation and ubiquitination, and highlight new roles for PTEN in protein trafficking in both cell lines and neurons. Building on these findings, further work will now be required to elucidate the mechanisms by which PTEN mediates glutamate receptor trafficking in neurons, and how these pathways may be perturbed in neurodegenerative diseases such as Alzheimer's.

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